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14. ABSTRACT Breast cancer patients exhibit high rates of late recurrent metastatic disease, which arise from tumor cells that lie dormant for extended periods before reawakening and developing into lethal metastases. We seek to delineate whether autophagy, a tightly controlled lysosomal digestion process, impacts the survival of dormant tumor cells, or alternatively, influences their ability to exit from dormancy and produce overt metastatic disease. Over the past year, we have obtained evidence using two immune competent mammary cancer models that autophagy inhibition promotes, rather than impedes, the development of overt metastasis in vivo. These findings suggest that autophagy restricts late recurrent disease by preventing the ability of dormant, disseminated tumor cells to exit from quiescent states and produce overt metastatic disease. Our initial mechanistic studies suggest multiple mechanisms by which autophagy restricts metastasis, including: 1) the cell autonomous control of focal adhesion signaling or proliferative outgrowth, or 2) the altered secretion of factors modulating efficient metastatic outgrowth and colonization. In parallel, we have created a polyoma middle T (PyMT)-based transgenic model exhibiting delayed kinetics with respect to the development of breast cancer macro-metastasis and one in which we can genetically ablate autophagy with both tumor cell and temporal/stage specificity. We are currently using this slow progression model to determine how to best modulate autophagy to prevent both metastatic colonization and late recurrence in vivo.

15. SUBJECT TERMS
breast cancer, mouse models, organotypic culture, autophagy, quiescence, dormancy

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I. INTRODUCTION:

Late recurrence continues to be a major barrier to eradicating breast cancer. Current evidence supports that such late recurrences in breast cancer likely arise from disseminated tumor cells that lie clinically dormant for extended periods of time [1]. These wayward breast cells either persist as solitary dormant cells, or form avascular micrometastases, which also remain dormant and clinically undetectable, presumably until they recruit appropriate micro-environmental factors to expand [1]. Nevertheless, to date, it remains largely unclear what biological processes govern the critical steps in late recurrent disease. These steps include: 1) the entry of breast cancer cells into dormancy, 2) the maintenance and survival of these cells during lengthy periods of quiescence, and ultimately, 3) their exit from dormancy to produce overt metastatic disease [2,3]. The overall goal of our research is to evaluate how changes in autophagy, a tightly-regulated lysosomal digestion process, impact one or more of these critical steps in the development of late recurrent breast cancer via the regulation of quiescent versus proliferative behavior exhibited by cancer cells. To address this issue, we have been taking several approaches in three-dimensional culture models and in vivo mouse transgenic model to assay both tumor dormancy and metastatic outgrowth in breast cancer.

During year 1 of this award, our 3D culture models uncovered that autophagy inhibition, achieved either via genetic loss-of-function of ATGs or via anti-malarials, promoted the active proliferation of quiescent cells [4]. During year 2, our in vivo models further suggested that autophagy inhibition promotes, rather than impedes the metastasis of polyoma middle T mammary cancers in vivo. These 3D culture and in vivo have unexpected implications for autophagy during late recurrent disease and suggests this process modulates the exit of dormant tumor cells from quiescent states to produce overt metastatic disease. Based on these results as well as other studies in years 1-2, we focused on the following goals and tasks during year 3 of the award: (1) We completed our studies of Ras/MAPK pathway activation in 3D culture models and published our findings in *Cancer Discovery*; this paper demonstrates a previously unrecognized, non-canonical function for autophagy in the coordinate production and secreted factors that promotes mammary cancer cell invasion and metastasis in vivo [5]. (2) We sought to corroborate our unexpected findings that autophagy suppresses macrometastatic outgrowth in the Polyoma middle T (PyMT) transgenic model using a second immune competent model of breast cancer metastasis—the 4T1 mammary carcinoma model [6]. These studies continue to support the hypothesis that that decreased autophagy promotes the exit of dormant tumor cells to produce overt metastatic disease. (3) Based on these results, we began studies to dissect the potential mechanisms by which autophagy suppresses metastatic colonization and outgrowth. (4) We continued to develop the compound transgenic strains in which autophagy can be modulated during PyMT cancer progression. We also initiated the experiments to evaluate the effects of autophagy ablation using this in vivo breast cancer slow progression model.

II. RESEARCH ACCOMPLISHMENTS BODY:

Task 1. Establish three-dimensional mammary epithelial cell culture and in vivo mouse model systems for late breast cancer recurrence.

a. Establish three-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane. (Months 1-12).

This sub-task was **completed** during year 1 and the findings were reported in the 2012 Annual Progress Report. Our studies of oncogenic-PI3K transformed breast cancer cells were published in *Oncogene* [4]*;* the full manuscript was included in the Appendix of the 2012 Annual Progress Report. Importantly, these data suggested that the inhibition of autophagy paradoxically promotes the active growth of quiescent cells, thus arguing against our originally proposed hypothesis.

b. Quantify rates of proliferation and apoptosis in human breast cancer cell lines grown in three-

dimensional epithelial organotypic cell culture (Months 1-24).

These studies were completed during years 1 and 2 and reported in the 2012 and 2013 Annual Progress Reports. *Overall, these studies revealed that two opposing, context-dependent functions for autophagy that will potentially influence late recurrent metastatic progression.* On the one hand, in breast tumors driven by the PI3K pathway, autophagy restricts proliferation and maintains a quiescent state. On the other, in tumors with hyperactivation of the Ras/MAPK pathway, autophagy has minimal effects on proliferation; rather, it promotes invasive behavior and alters epithelial differentiation and secretion. Our in vivo studies have been focused on precisely defining and elaborating these two opposing biological functions in vivo during analysis of the autophagy pathway in the transgenic breast models we have generated.

As reported in the 2013 Annual Report, one of our major accomplishments from sub-tasks 1a and 1b was the discovery of a new cell biological function for autophagy during recurrent disease and metastasis. Our conditioned media experiments in 3D culture demonstrated that autophagy-deficient Ras cells, fail to secrete pro-invasive factors. We also discovered that reduced autophagy specifically diminishes the secretion of the pro-migratory cytokine, interleukin-6, which is necessary and sufficient to restore invasion of autophagy-deficient cells. **Over the course of this reporting year, we submitted these experimental findings for publication, which were published in the prestigious journal *Cancer Discovery* in 2014 (see Appendix)** [5]. Importantly, based on peer review comments for this manuscript, we conducted additional experiments over the past year in order to further solidify and expand the conclusions we had proposed based on the original findings in tasks 1 and 2. The salient experiments are delineated below.

First, we developed methods to quantify the invasive behavior observed during the conditioned media experiments in autophagy competent and autophagy-deficient (shATG7 or shATG12) cultures of $HRas^{V12}$-transformed mammary epithelial cells (Fig 1). For these quantifications, we adapted a published technique developed by the lab of Senthil Muthuswamy (Ontario Cancer Institute) to quantify the percent of invasive protrusions documented via phase microscopy [7]. To utilize this metric, we analyzed cultures at 48 hours following conditioned media (CM) treatment in which invasive protrusions emanating from individual

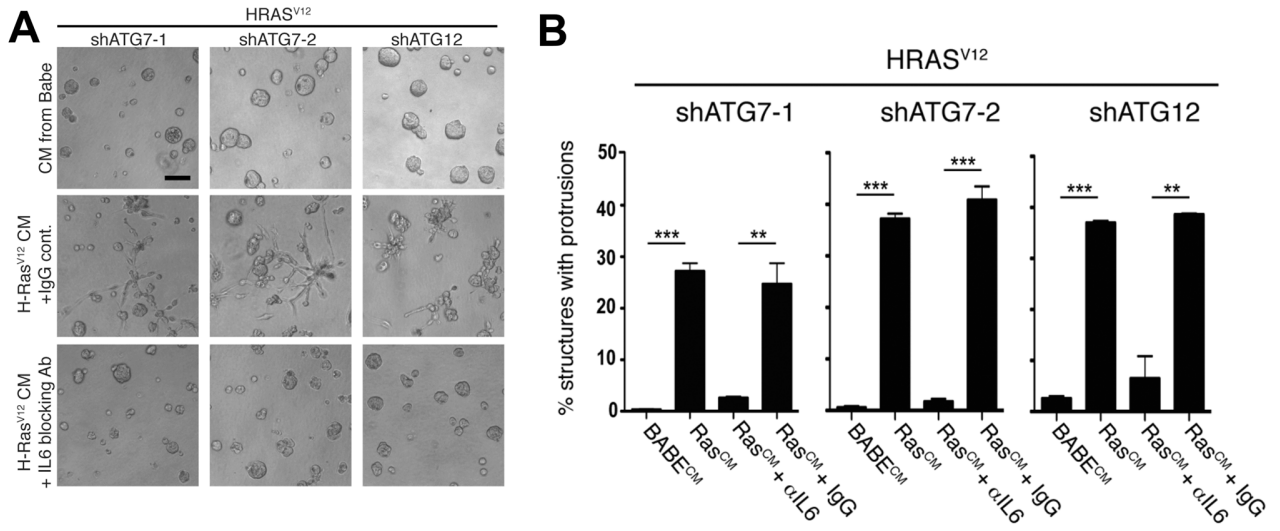


Fig. 1: Quantification of IL6-mediated invasion in 3D culture. (A) Representative phase contrast images of H-RasV12 MCF10A shATG 3D cultures treated for 48h with nontranformed (BABE) CM (top) or with H-RasV12 transformed CM containing an IL-6 function blocking antibody (bottom) or IgG control antibody (middle). Bar, 100 μm. (B) Quantification of invasive protrusions in $HRAS^{V12}$ shATG cells following treatment with the indicated conditioned media (CM) for 48h; the percent of structures in a culture with one or more invasive protrusion was enumerated. **P<0.01, ***P<0.001 (Student's t test, n=3).

structures were present but complex intermingled networks had not formed. The quantification of nontransformed control (BABE) vs. HRas^{V12} CM addition and IL6 function blocking results are presented in the graphs in Fig 1B. They demonstrate a 30-40 fold increase in invasive protrusions in shATG cultures treated with CM produced from HRas^{V12}-transformed cells compared to that produced from nontransformed cells. Furthermore, the addition of a function-blocking antibody against IL6 results in the near complete reversal of this phenotype, whereas incubation with an isotype control (IgG) has little effect. Overall, these quantifications corroborate our original conclusions that we presented in the 2013 Annual Progress Report that interleukin-6 (IL6) produced via autophagy-competent cells is a critical factor necessary for invasion.

Second, we more rigorously evaluated whether IL6 re-addition was truly sufficient for invasion by HRas^{V12} shATG cells grown in three-dimensional culture. As shown in Fig 2A, we evaluated the effects of recombinant IL6 on basement membrane integrity, because the disruption of intact basement membranes are a hallmark of breast carcinoma invasion *in vivo* [8]. To delineate the basement membrane, we examined the localization of the basement membrane protein laminin 5 in H-Ras^{V12}-derived autophagy-deficient cultures in the presence versus absence of recombinant human IL6 (rhIL6). Consistent with our previous results, individual structures in ATG deficient H-Ras^{V12} cultures were encompassed by an intact basement membrane, indicating that ATG knockdown restored polarized basement membrane secretion. In contrast, upon addition of rhIL6, we observed the cytosolic accumulation of laminin 5, which was especially prominent in the invasive protrusions of H-Ras^{V12} shATG cultures, with no evidence of polarized deposition at the cell-ECM interface. In addition, using the methods described above, we quantified the effects of IL6 re-addition on the invasive capacity of H-Ras^{V12} shATG cells (Fig. 2B) and demonstrated a 20-30-fold increase in invasive protrusions. These results supported that IL6 was sufficient to restore the invasive capacity of autophagy-deficient cells and corroborated this cytokine as a key pro-invasive factor regulated via an intact autophagy pathway.

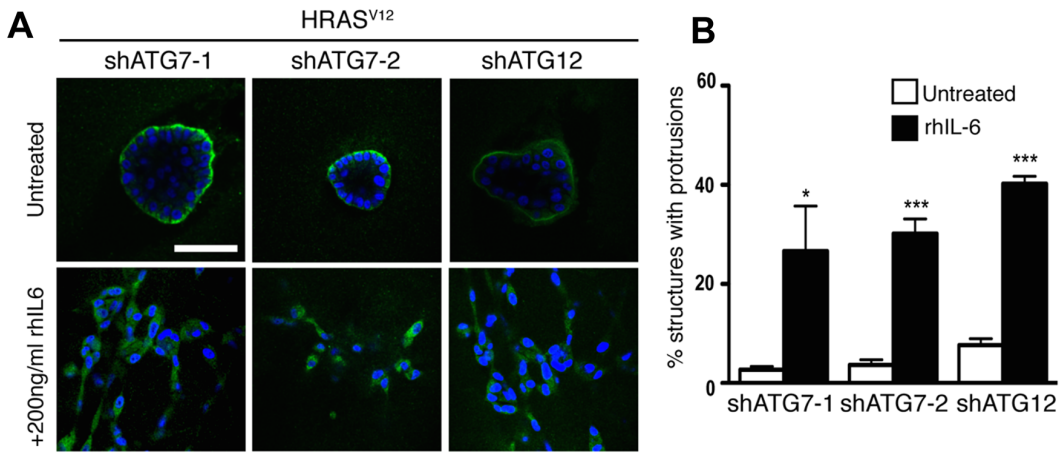


Fig. 2: Effects of recombinant human IL6 re-addition on HRAS^{V12} shATG 3D cultures. (A) 3D cultures of HRAS^{V12} shATG cells were treated with 200ng/ml rhIL6, fixed and immunostained with an antibody against LAMA5 and DAPI counterstained. Bar, 50 μm. (B) Quantification of invasive protrusions in HRAS^{V12} shATG cells following treatment with 200ng/ml rhIL6; the percent of structures in a culture with one or more invasive protrusion was enumerated. *P<0.05, ***P<0.001 (Student's t test, n=3).

Third, we interrogated whether autophagy-dependent secretion of IL6 was sufficient to promote invasion of non-transformed cells (BABE) grown in 3D culture. However, neither the addition of conditioned media produced from H-Ras^{V12}-transformed cultures nor rhIL6 itself was sufficient to promote 3D invasion by non-oncogenic cells (Fig. 3A, B). Thus, autophagy-dependent secretion promotes invasion in the context of oncogenic Ras pathway activation.

Although rhIL6 addition was able to restore invasive protrusions in H-Ras^{V12} shATG cultures, we noted that the ATG knockdown structures did not form intermingled networks to the same extent as treatment with H-

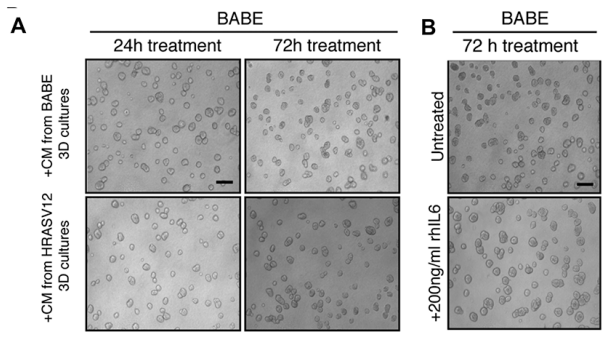


Fig 3. Effects of conditioned media treatment and recombinant human IL6 re-addition non-transformed MCF10A 3D cultures. (A) Nontransformed MCF10A cells expressing empty vector (BABE) were cultured in 3D for 3d and subsequently treated with BABE or HRAS^{V12} shCNT conditioned media (CM). Representative phase contrast images at 24h and 72h following the addition of CM. Bar, 100 μm. (B) Representative phase contrast images of BABE 3D cultures grown in the presence or absence of 200ng/mL recombinant human IL6 (rhIL6). Bar,100 μm.

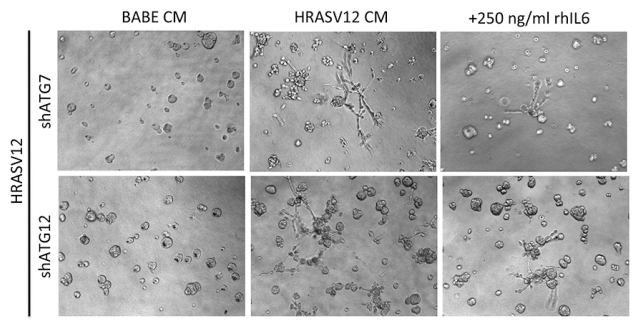


Fig 4: HRAS^{V12} conditioned media restores invasive networks in HRAS^{V12} ATG knockdown cells more potently than IL6. HRAS^{V12} shATG cells grown in 3D culture for 4 days were treated for 48h with CM from non-transformed (BABE) cultures (right), HRAS^{V12}-transformed cultures (middle), or 250ng/ml rhIL6 (right). Representative phase contrast images are shown.

Ras^{V12} conditioned media (Fig 4). As a result, we interrogated whether autophagy modulated the production of additional secreted factors favoring invasion. We performed a qPCR array to measure the expression levels of genes involved in EMT and invasion, and identified *WNT5A* and *MMP2* as two candidate factors whose expression was upregulated in HRAS^{V12} cells relative to BABE cells but potently suppressed upon autophagy inhibition. qPCR analysis of cells collected from 3D cultures confirmed a 2-fold decrease in *mmp2* and *wnt5a* expression in HRAS^{V12} shATG cells compared to HRAS^{V12} shCNT (Fig. 5A and B).

Because these secreted factors have been implicated in cell migration and invasion, we further evaluated whether their decreased expression following ATG knockdown also contributed to the reduced invasive potential of HRAS^{V12} shATG cells. First, we utilized gelatin zymography to assess MMP2 activity in CM from 3D cultures. MMP2 activity was enhanced in HRAS^{V12} cells compared to non-transformed (BABE) controls, and upon ATG knockdown in HRAS^{V12} cells, this activity was reduced (Fig. 5C). The increase in MMP2 expression and secretion following constitutive RAS activation was necessary for RAS-driven invasion, as addition of an MMP2 inhibitor, Arp-100, was sufficient to inhibit the formation of invasive protrusions in HRAS^{V12} 3D cultures (Fig. 5D). Furthermore, the decrease in *wnt5a* expression correlated with a decrease in WNT5A protein levels in HRAS^{V12} shATG cells isolated from 3D culture (Fig. 5E). Moreover, the addition of recombinant WNT5A to HRAS^{V12} shATG7-1 3D cultures promoted the dissociation of cells within the structures and enhanced the formation of invasive protrusions (Fig. 5F). Thus, in addition to IL6, autophagy facilitates the production of multiple secreted pro-migratory and invasive factors that support RAS-driven invasion in 3D culture.

In contrast to reduced IL6 secretion, which is likely a more proximal event following ATG knockdown, these changes in WNT5A and MMP2 result from decreased gene expression, indicating that autophagy inhibition produces broader transcriptional changes contributing to reduced invasion by HRAS^{V12} cells. Accordingly, we analyzed whether IL6 was responsible for these transcriptional changes by testing whether rhIL6 addition was sufficient to restore their expression in autophagy-deficient cultures. However, as shown in Fig. 6A, we did not find any differences in rhIL6-treated versus untreated cells, indicating the regulation of these factors is IL6 independent. Moreover, rhIL6 only partially rescued the effects of autophagy-deficiency on epithelial differentiation (Fig. 6B). Overall, these results support that autophagy in promoting cancer cell invasion via the coordinate production of multiple secreted factors in addition to IL6. In the upcoming years, we will investigate how these factors impact late recurrent disease and metastatic outgrowth in vivo as part of our studies in task 2.

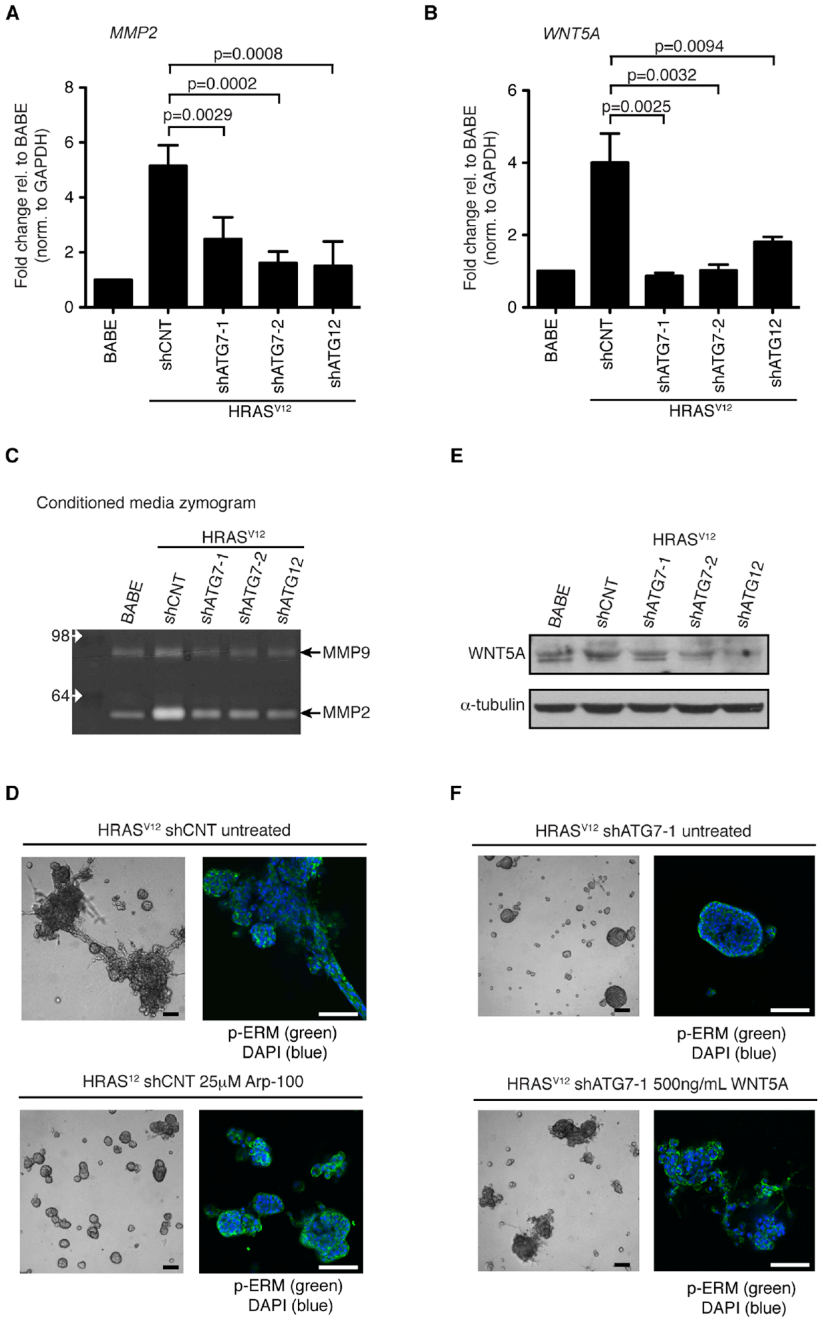


Fig. 5. WNT5A and MMP2 are reduced following autophagy inhibition in 3D culture. (A-B) RNA was isolated from BABE, HRAS^{V12} shCNT, and HRAS^{V12} shATG cells cultured in 3D for 8 days. Expression levels of *MMP2* and *WNT5A* were determined by qPCR and normalized to an internal control *GAPDH*. Results represent the mean relative to BABE +/- s.d. (*MMP2,* n=4; *WNT5A*, n=3; Student's t-test). (C) Conditioned media was collected from BABE, HRAS^{V12} shCNT and HRAS^{V12} shATG cells grown in 3D culture. Activity levels of MMP9 and MMP2 in the conditioned media were determined by zymography. (D) HRAS^{V12} shCNT cells were grown in the absence (top) or presence (bottom) of 25mm Arp-100. Left: Structures were imaged on day 8 by phase contrast microscopy. Right: Representative confocal images of structures immunostained with anti-phospho-ERM (P-ERM) to detect cell borders and counterstained with DAPI. Bars, 100μm. (E) BABE, HRAS^{V12} shCNT, and HRASV12 shATG cells were collected from 3D culture on day 8, lysed, and protein levels of WNT5A were determined by immunoblot analysis. (F) HRAS^{V12} shATG7-1 cells were grown in 3D for 8 days in the absence (top) or presence (bottom) of 500ng/mL WNT5A. Left: Representative phase contrast images. Right: Representative confocal images immunostained with anti-phospho-ERM to detect cell borders. Bars, 100μm.

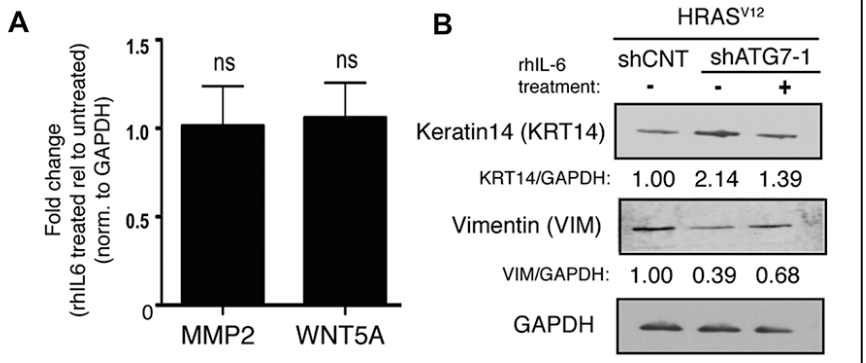


Fig. 6: Effects of rhIL6 treatment on *MMP2* and *WNT5A*. (A) Fold change in *MMP2* and *WNT5A* expression in cells isolated from $HRAS^{V12}$ shATG7-1 3D cultures following treatment with 200ng/ml rhIL6. Results represent the fold change relative to untreated cultures (mean +/- s.d.). ns, nonsignificant. (*MMP2,* n=4; *WNT5A*, n=4, Student's t-test). (F) Effects of rhIL6 treatment on keratin 14 (KRT14) and vimentin (VIM) protein levels in $HRAS^{V12}$ shCNT and $HRAS^{V12}$ shATG7-1 cells grown in 3D culture in the presence or absence of 200ng/mL recombinant human IL6 (rhIL6).

c. Optimize protocols for the stable ex vivo transduction of fluorescent (e.g., GFP), luminescent (e.g. luciferase), and drug resistant (e.g., puromycin) marker proteins into human cancer cell lines. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols were detailed in the 2012 Annual Progress Report.

d. **For human cells in subtask 1b that exhibit a low proliferation index in 3D culture, introduce cells (containing fluorescent and/or drug resistance marker proteins) into the systemic circulation of immunodeficient mice and determine the latency period to the onset of metastasis. (Months 13-36; Revised timeline: Months 37-60).**

Based on our studies in subtask 1b during years 1 and 2, we identified the luminal breast cancer lines MCF7 and T47D as the most useful for 1d and 1e. Because of the unexpected results regarding autophagy that we obtained in our 3D culture studies, as well as in vivo results from PyMT and 4T1 cells described in subtask 2b, we deferred the initiation of these studies until we obtained more robust data from the PyMT model in sub-tasks 2b-c in order to prioritize the studies from PyMT tumors regarding late recurrent disease because it is an immune competent, syngeneic model. The initial results are described below and support that autophagy impedes, rather than promotes, late recurrent growth. We are continuing these studies using the immune competent models we have developed. Based on those studies, we will revisit salient issues using the immunodeficient models in this task; we anticipate that the MCF7 cell line, which harbors an oncogenic PI3K mutation, will be the most appropriate human cell line to confirm and extend if autophagy impedes late recurrent growth in the PyMT model.

e. Isolate late onset macro-metastatic tumors and dormant tumor cells from subtask 1d, and obtain gene expression profiles. To obtain dormant cells from each cohort in subtask 1d, five to seven (5-7) tumor cell bearing mice will be euthanized at an intermediate time point (anticipated to occur at six to nine (6-9) months post initial injection but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 48-60).

These studies have not been initiated. Because subtask 1d is being delayed to pursue studies in the PyMT model, we have revised the timeline for subtask 1e accordingly.

f. Generate transgenic mice expressing MMTV-PyMT (Polyoma Middle T) and ROSA26-LSL-RFP in a pure genetic background (C57B/6). (Months 1-12).

We completed the generation of these compound transgenic mice in a pure C57B/6 genetic background, which was described in the 2012 and 2013 Annual Progress Reports.

g. Establish routine isolation and short-term culture conditions for normal and neoplastic mouse mammary epithelium. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols have been detailed in the 2012 Annual Progress Report.

h. Optimize protocols for the stable ex vivo transduction of Cre recombinase and fluorescent, luminescent, and/or drug selection marker proteins into normal and neoplastic mouse mammary epithelium. (Months 1-12).

This sub-task was **completed** during year 1. The optimized protocols have been detailed in the 2012 Annual Progress Report.

i. Isolate neoplastic epithelium from female transgenic mice generated in subtask 1f and transplant into syngeneic C57B/6 host recipient animals. (Months 13-24).

This sub-task was completed during year 2 and reported in the 2013 Progress Report.

j. Determine the latency period to the onset of metastasis for recipient mice generated in subtask 1i. Fifteen mice (15) will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 25-36; Revised timeline: Months 25-48).

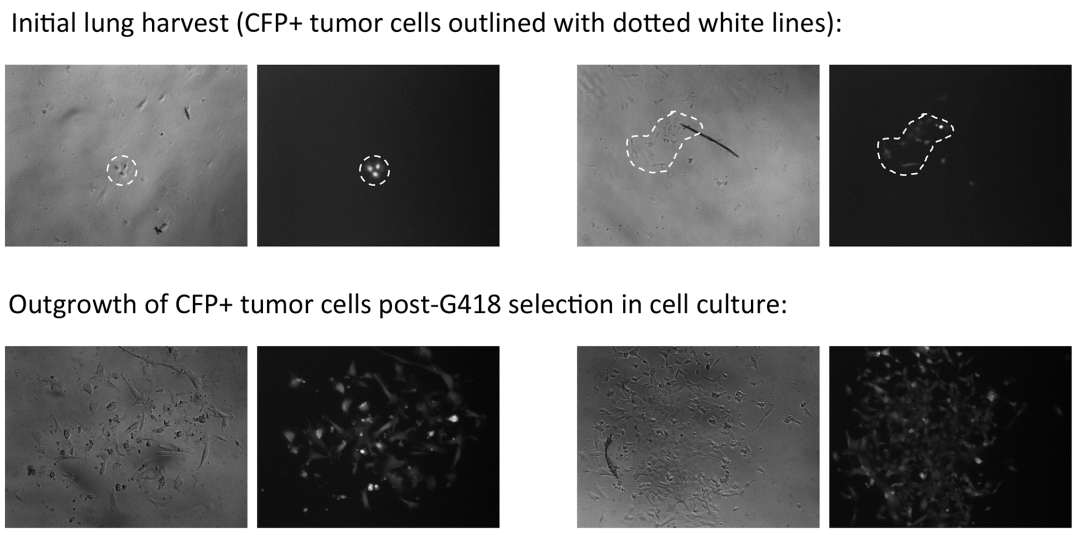


Fig. 7: Isolation of disseminated tumor cells from a spontaneous metastasis assay. MMTV-PyMT, β-actin CFP tumors were transplanted into the mammary fat pad of C57B/6 recipient mice. Top: Lungs were harvested and minced to generate single cell suspensions; CFP positive tumor cells were detected within these suspensions. Bottom: Tumor cells expressing CFP were cultured out of harvested lung tissue. Tumors are neomycin (G418) resistant due to the MMTV-PyMT transgenic cassette; hence, G418 selection results in the preferential enrichment of disseminated tumor cells.

We analyzed a pilot cohort (n=5) transplanted with PyMT cells for 120 days (6 months). Although we did not detect overt tumors in intact lung tissue, we generated single cell suspensions from these lungs and via immunofluorescence microscopic analysis. We detected isolated CFP-positive tumor cells residing within the lung tissue of recipient mice; moreover, upon selection with G418, we observed the outgrowth of CFP-positive PyMT tumor colonies ex vivo, thereby confirming the presence of disseminated tumor cells (Fig. 7). This tumor cell seeding suggests that metastatic disease will potentially develop over a longer time period. Currently, we are transplanting tumors into a larger cohort (n=10) of syngeneic C57B/6 host recipient

animals and will await the development of overt, metastasis for these studies in subtask 1j. Consistent with our goal to generate a model of late recurrence, we anticipate the onset of metastasis will occur over an extended time course; our experimental plan is to sacrifice these mice at two later time points—8 months and 10 months (n=5 mice for each time point).

k. Isolate late onset macro-metastatic tumors and dormant tumor cells from mice in subtask 1i, and obtain gene expression profiles. To obtain dormant cells from subtask 1i, ten (10) tumor cell bearing mice from 1i will be euthanized at an intermediate time point (anticipated to occur at six to nine (6-9) months post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 37-48).

These studies have not been initiated. We will conduct the studies over the upcoming year if we are able to obtain overt lung metastasis through the studies in task 1j.

Task 2. Determine the requirement for autophagy in the survival of dormant breast cancer cells.

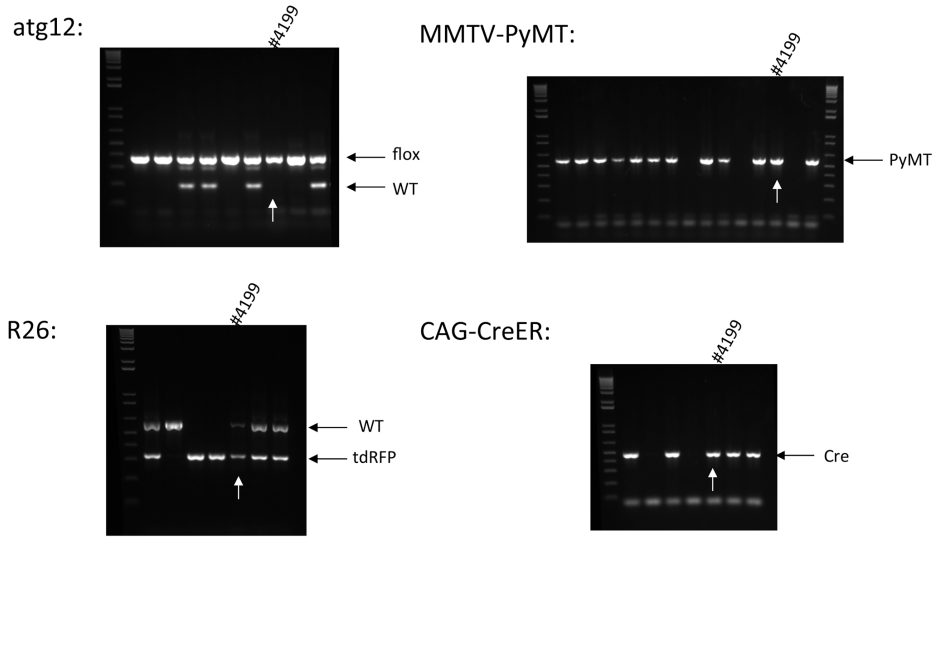
a. Generate transgenic mice expressing MMTV-PyMT (Polyoma Middle T), ROSA26-LSL-RFP, and conditional null alleles (floxed) of autophagy regulators (e.g., atg12 and atg5) in a pure genetic background (C57B/6). (Months 1-24).

Table 1: Genotypes of PyMT Donor Epithelium

Date Frozen	Tumor (Mouse ID)	Genotype
11/10/2012	2385	atg12 f/f; MMTV-PyMT+
11/15/2012	2446	atg12 f/f; MMTV-PyMT+; β-actin-CFP+
1/23/2013	2848	atg12 f/f; R26-tdRFP+/+; MMTV-PyMT+
2/20/2013	2800	atg12 f/f; R26-tdRFP+/-; MMTV-PyMT+; β-actin-CFP+
2/20/2013	2938	atg5 f/f; MMTV-PyMT+
4/29/2013	3259	atg12 f/f; R26-tdRFP+/+; MMTV-PyMT+; β-actin-CFP+
5/6/2013	3306	atg5 f/f; MMTV-PyMT+; β-actin-CFP+
6/10/2013	3305	atg5 f/f; MMTV-PyMT+; β-actin-CFP+
6/27/2013	3529	atg12 f/f; R26-tdRFP+/-; MMTV-PyMT+; WAP-rtTA-Cre+
8/12/2013	3799	atg12 f/f; R26-tdRFP+/+; MMTV-PyMT+; WAP-rtTA-Cre+
11/13/2013	3846	atg5 f/f; MMTV-PyMT+
11/24/2013	4199	atg12 f/f; R26-tdRfP+/-; MMTV-PyMT+; CAG-CreER+
12/12/2013	4388	atg12 f/f; MMTV-PyMT+; β-actin-CFP+
4/16/2014	4858	atg5 f/f; MMTV-PyMT+; β-actin-CFP+
6/25/14	5538	atg12 f/f; MMTV-PyMT+; CAG-CreER+

We completed the interbreeding of the mice to generate the compound transgenic mice necessary for these studies, which we described in the 2013 Annual Progress Report. Based on the protocols to harvest and freeze down tumor tissue for cryostorage in subtask 1g (detailed in the 2012 Annual Progress Report), we have continued to harvest tumors from donor animals for our transplantation studies over the past year. Table 1 summarizes the genotypes for the key tumors we have generated from donor animals over both years 2 and 3. All of the animals in Table 1 are in a C57B/6 pure genetic background and suitable for our transplantation strategies. These isolated cells from these donor tumors can be stored for extended periods, thawed, expanded and reintroduced into syngeneic recipient animals at a later date.

Importantly, over the past year, we generated a new strain of animals to delete autophagy in tumor cells in an inducible manner. The creation of this strain became a high priority over the previous years because our studies using the PyMT-R221 and 4T1 cell-based models described below in sub-task 2b suggested that autophagy may be impeding a very specific step in the early metastatic colonization cascade. These



Mouse #4199: atg12 f/f; R26-tdRFP+/-; MMTV-PyMT+; CAG-CreER+

Fig 8: Genotyping of mice expressing *atg12* floxed alleles and transgenes encoding MMTV-PyMT, ROSA26-LSL-RFP, and CAG-CreER. Separate PCR reactions were used to detect the ATG12 floxed alleles and each of the indicated transgenic alleles from tail DNA. Mice 4199, marked by white arrows on the gels, is homozygous for the *atg12* floxed allele (*atg12f/f*) and also positive for MMTV-PyMT, ROSA26-LSL-RFP, and CAG-CreER.

compound transgenic mice contain MMTV-PyMT, *atg12* floxed alleles, tamoxifen-inducible Cre recombinase (CAG-Cre^{ERT}) [9], as well as a fluorescent reporter (R26-LSL-RFP) to monitor Cre excision [10] (Fig. 8). Upon harvesting tumors from these mice, we have confirmed efficient ATG12 deletion ex vivo upon treatment with 4-hydroxytamoxifen (4OHT) (Fig. 9). Moreover, upon ATG12 deletion, we have confirmed the complete ablation of autophagy, evidenced by the lack of LC3-II and the accumulation of p62/SQTM1, a protein selectively degraded by autophagy (Fig. 9). To control for the effects of 4OHT-mediated Cre expression, we possess control mice with MMTV-PyMT, CAG-Cre^{ERT} and R26-LSL-RFP, but wild type for *atg12*. Overall, subtask 2a has been successfully completed and we now possess a bank of PyMT tumor tissue in which we can genetically ablate autophagy in a tumor cell specific manner for our proposed subtasks in the upcoming years; moreover, in the case CAG-Cre-ER animals, we can also conduct these studies of ATG deletion in a temporal-specific manner if the need arises.

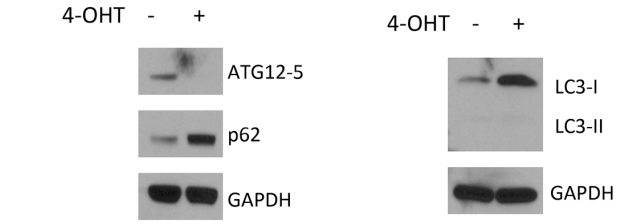


Fig 9: Inducible ATG12 deletion in primary PyMT organoids following 4OHT treatment ex vivo. Immunoblotting of lysates derived from these cells confirms complete loss of the ATG12-ATG5 complex and autophagy ablation, based on the accumulation of p62/SQSTM1 as well as the loss of PE-lipidated LC3 (LC3-II).

b. Using techniques optimized in subtasks 1g-i, isolate neoplastic epithelium from female transgenic mice generated in subtask 2a, transduce with Cre recombinase, and transplant into cleared mammary fad pads of syngeneic C57B/6 host recipient animals. For this subtask, we anticipate that

at least ten (10) mice from each transgenic donor will be utilized for epithelial isolation and at least twenty five (25) host recipient animals per cohort will be utilized for fat pad transplantation (Months 19-36; revised timeline: 19-48).

We are utilizing the primary PyMT mouse models generated in sub-task 2a to ascertain whether autophagy similar impacts metastatic outgrowth in a slow progression model to better model late recurrent disease. We recently transplanted C57B/6 hosts with autophagy-competent (*atg12* WT) and autophagy-deficient PyMT cells (*atg12* deleted), using 10 mice per cohort for the first study; because we have not observed overt macro-metastasis in our studies of wild type, autophagy competent control PyMT cells in subtask 1j, we will continue this experiment over a 10-12 month time course extending into the upcoming year for the studies in sub-task 2c. We anticipate the ongoing studies in subtask 1j will provide guidance in determining the optimal time point to analyze these mice. Because of our results to date, described in sub-task 2c below, we will complement these ongoing experiments using spontaneous metastasis assays with experimental metastasis assays in order to ascertain the specific roles of autophagy-deficiency during early lung colonization.

Furthermore, as we reported in the 2013 Progress Report, we obtained an unexpected finding that the genetic inhibition of autophagy led to increased, rather than decreased, rates of metastasis in the lung using a more rapid progression model-PyMT-R221 tumor cell model. We continued the analysis of these mice and extended these findings into a second immune-competent mouse model-4T1. *The results from these experiments performed over the last year are described in detail in sub-task 2c below.* Given the unexpected results we obtained with the more rapidly progressing PyMT-R221 and 4T1 models over the last 2 years, we placed increased priority on generating the MMTV-PyMT, atg12f, LSL-RFP, CAG-Cre^{ERT} model, as described in sub-task 2b. With the successful generation of this compound transgenic strain (Figs. 8 & 9), we plan to transplant these cells into syngeneic host animals in the upcoming year.

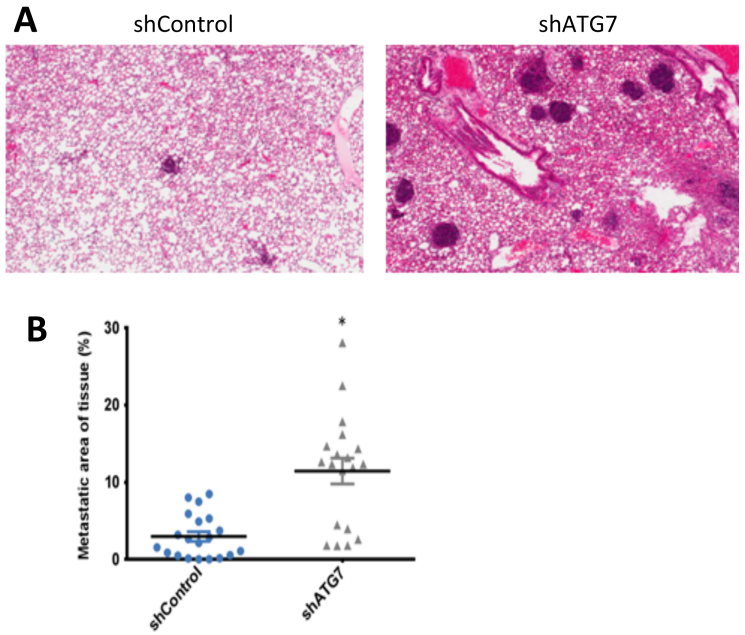


Fig. 10: Increased lung metastasis following ATG7 knockdown in PyMT-R221A cells. (A) (A) H&E staining of lungs. (B) Quantification of percentage area occupied by metastatic tissue. Data is from 3 independent experiments n=20 mice for shCTRL and n=19 mice for shATG7.

c. Determine whether the deletion of autophagy regulators from transplanted neoplastic breast cells affects the onset of metastasis in recipient mice from subtask 2b. Fifteen (15) mice from each cohort will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 25-48).

In year 2, we made the unexpected finding that the genetic inhibition of autophagy led to increased, rather than decreased metastasis in the lung; the initial findings were reported in our 2013 Annual Progress Report. To summarize, these studies were conducted using the PyMT based model in which tumor cells were transplanted into syngeneic hosts for analysis of metastasis phenotypes. To

inhibit autophagy, we expressed shRNA against ATG7 (shATG7) for stable RNAi-mediated silencing of this essential autophagy regulator. Using both experimental and spontaneous metastasis models, we discovered that mice transplanted with autophagy-deficient PyMT cells exhibited a significantly increase in overall number of macrometastases.

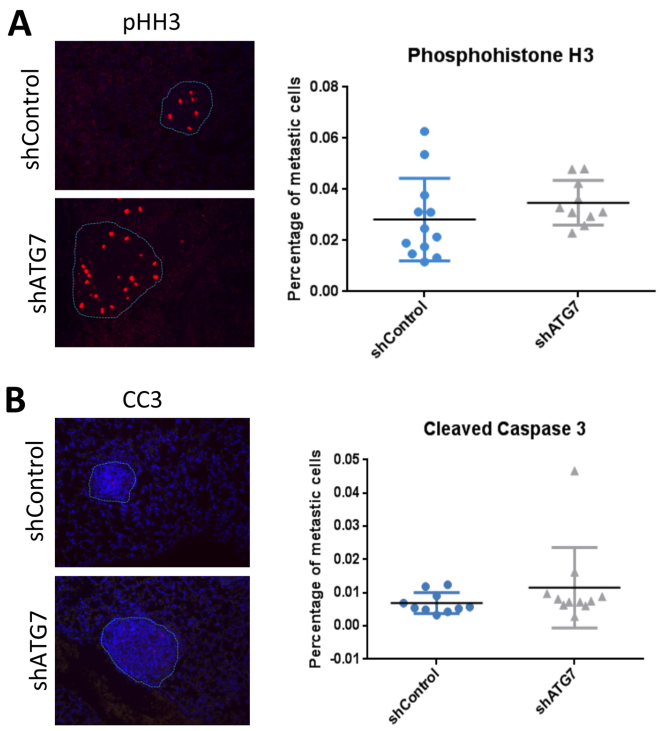


Fig 11: Autophagy inhibition does not affect proliferation or apoptosis within overt metastasis. Lung metastasis in PyMT-R221 mice were immunostained with antibodies against: (A) phospho-histone H3 pHH3) to detect proliferating cells and (B) cleaved caspase-3 (CC3) to detect apoptosis. Upon quantification, no significant differences were found in the rates of proliferation and apoptosis in the macrometastatic lesions from these mice. Data is from 3 independent experiments n=10 mice for shCTRL and n=9 mice for shATG7.

During the last year, we further analyzed this experiment and corroborated that the lungs of PyMT-shATG7 tumor bearing animals exhibited an approximately 2-fold increase in metastatic burden, based on the analysis of the percent total area of the lung occupied by metastatic tumor (Fig 10A-B). Moreover, we analyzed the rates of proliferation and apoptosis in metastatic lesions using the mitosis marker, phospho-histone H3 pHH3) to detect proliferating cells as well as cleaved caspase-3 (CC3) to detect apoptosis (Fig 11A-B); however, we did not find any significant differences between autophagy-competent versus autophagy-deficient cells macrometastatic lesions with respect to either of these parameters. These findings suggest that autophagy may be restricting an earlier step in the metastatic cascade, either the efficiency of seeding at the foreign site or an early step in the colonization process.

Based on this initial analysis, an important priority became to corroborate these findings in an independent model. We chose to do so using second well-established immune competent model of mouse mammary carcinoma progression and metastasis-the 4T1 model. Similar to PyMT, the 4T1 mammary cancer model exhibits high rates of metastasis to distant sites, including the lung, with established kinetics [6]. Using lentiviral RNAi, we achieved stable, robust autophagy inhibition in these cells via shRNA knockdown of ATG7 or ATG12, two essential components of the autophagy core machinery, evidence by the loss of PE-lipidated LC3 (LC3-II) (Fig. 12A-B). Upon orthotopic transplantation into syngeneic BALB/c hosts, primary 4T1 tumors rapidly grow over a 3-4 week period. Consistent with long-term autophagy inhibition, ATG7/12 deficient tumors exhibited the loss of LC3-II and the accumulation of p62/SQSTM, an autophagy cargo adapter degraded via autophagy (Fig. 12C)[11]; however, no differences in primary tumor growth were observed (Fig. 13). Nevertheless, the initial analysis of the lungs revealed increased metastasis in animals transplanted with ATG-deficient tumors compared to controls; the size of individual metastatic deposits in the lung was larger and the numbers of metastasis was increased (Fig. 14). Overall, these results verify that autophagy impedes rather than promotes metastasis in two established immune-competent models of breast cancer metastasis in vivo—4T1 and PyMT.

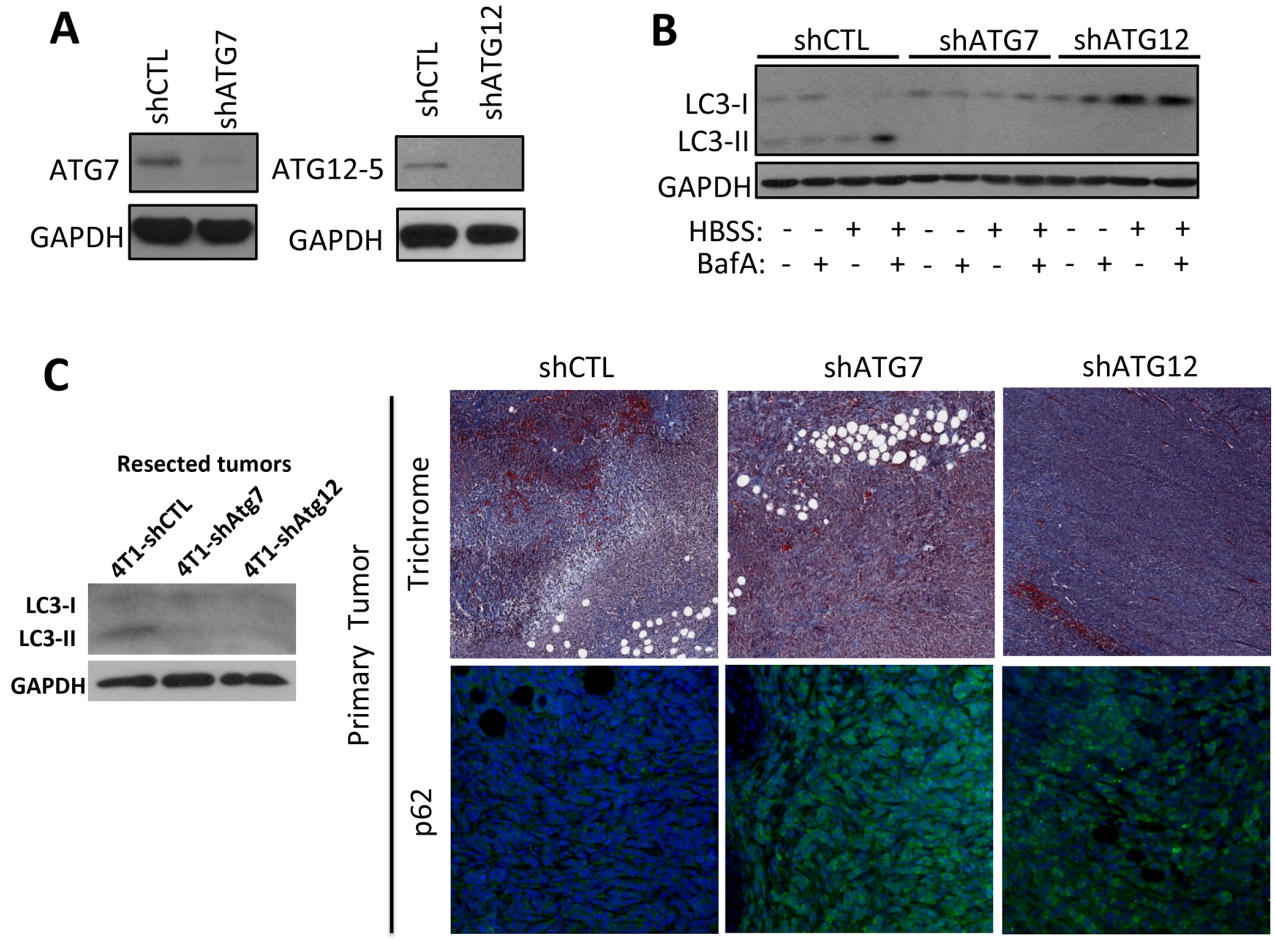


Fig. 12. ATG knockdown in 4T1 mouse mammary cancer cells. (A) Verification of shRNA-mediated silencing of ATG7 or ATG12 in 4T1 cells. (B) ATG7/12 knockdown impairs autophagic flux in 4T1 cells following HBSS-mediated nutrient starvation in vitro. The lysosomal turnover of LC3-II was evaluated in the presence versus absence of 10nM Bafilomycin A (BafA). (C-D) Orthotopic shATG primary tumors continue to exhibit reduced LC3-II and accumulate p62/SQSTM1.

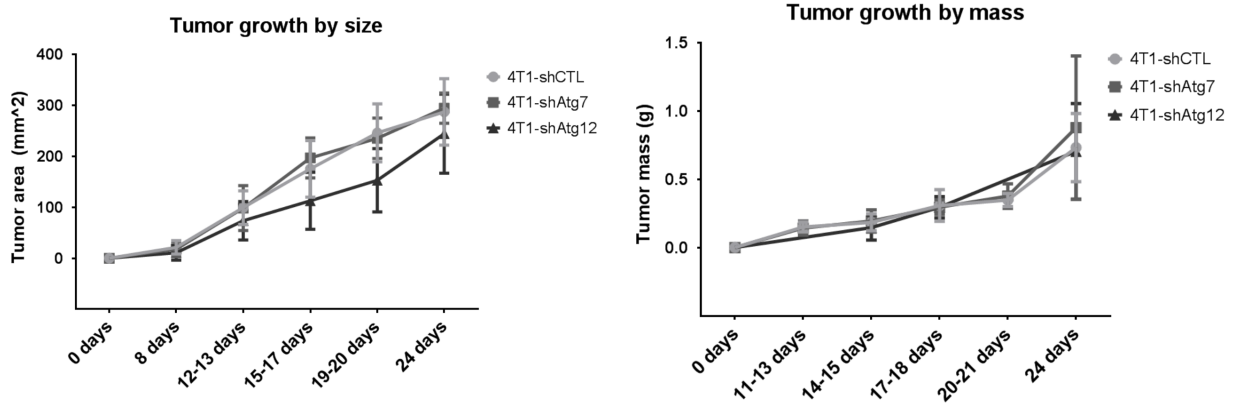


Fig 13: Autophagy deficiency does not affect growth of 4T1 primary tumors. Mice were orthotopically transplanted with 4T1 cells with the indicated shATGs and tumor size was measured using calipers (left) or mice were euthanized and the tumor weight was recorded. N=10-12 tumors were analyzed for each cohort.

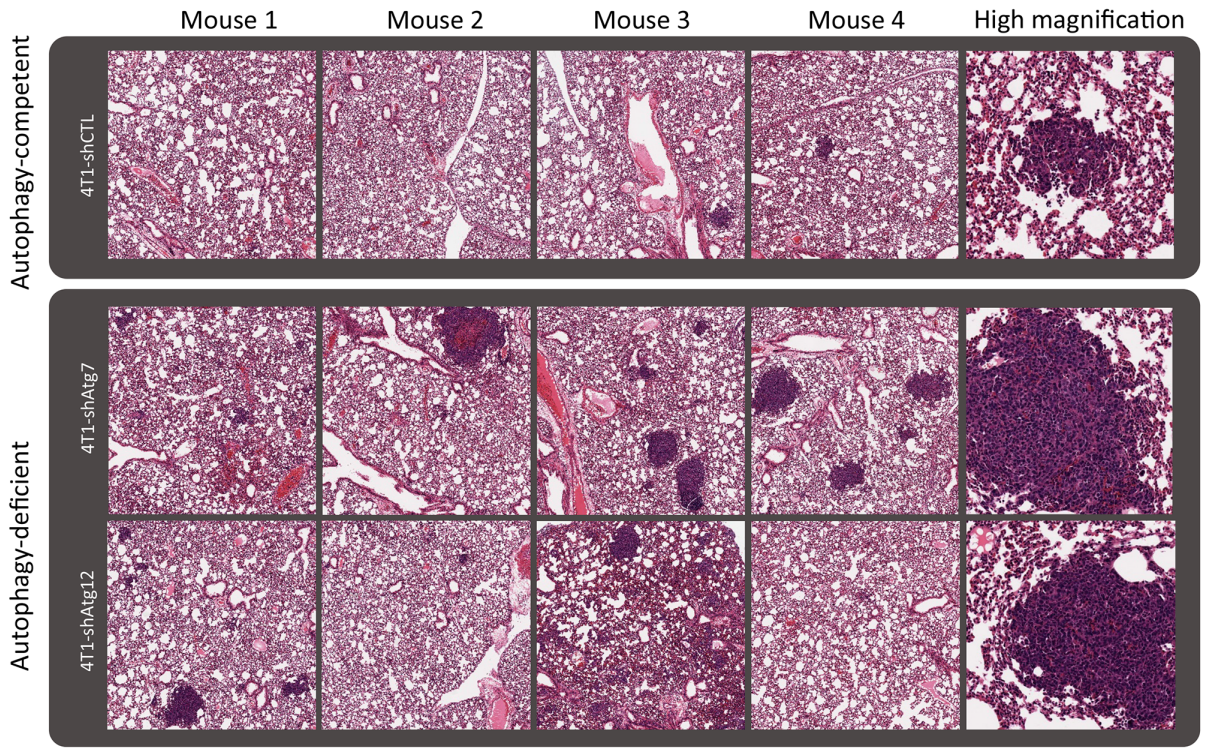


Fig 14: ATG knockdown in 4T1 mouse mammary cancer cells enhances spontaneous metastasis to lung. Mice were orthotopically transplanted into the mammary fat pad with 4T1 cells that were autophagy-competent (shCTL) and autophagy-deficient (shATG7 or shATG12). Lungs were harvested at 21 days post-transplant. H & E staining of lung tissue reveals increased numbers of metastasis and increased size of metastatic lesions in autophagy-deficient cells. 4 individual mice are show; quantification of metastatic burden is still in progress.

Notably, these findings in both mammary cancer models contrast with our recently published results from studies in sub-task 1b conducted using HRasV12-transformed cells and reported in the 2012 Annual Progress Report [5]. Several reasons may explain these differences. First, since Ras-transformed cells are highly dependent on autophagy for growth and metabolism, the effects on autophagy inhibition on metastasis may be unique to this oncogenic context [2]. Second, our previous studies were limited by the use of immune-compromised, rather than, immune-competent, models. Third, and most importantly, autophagy may suppress metastasis at a specific stage, most notably early colonization at the foreign tissue site, which is viewed by the cancer biology field as a rate-limiting step in metastasis[12]. This has clinical implications because it suggests that enhanced metastasis may be a long-term risk of autophagy inhibitors like hydroxychloroquine (HCQ). Thus, a key unresolved issue is resolving the mechanisms by which reduced autophagy suppresses early metastatic colonization in breast cancer. We have started to address this critical gap in knowledge through the mechanistic studies described below.

Pro-metastatic functions of autophagy cargo receptors: Autophagy cargo adaptors, such as p62/SQSTM1 mediate selective autophagic degradation of intracellular proteins, and in the process, they are degraded via autophagy [11]. In cancer, the accumulation of autophagy cargo adapters, specifically p62/SQSTM1, has been shown to drive primary tumor growth in autophagy-deficient cells [13-16]. Notably, during the first year of this award, we demonstrated in sub-task 1a that p62 accumulation was a key mediator of oncogenic PI3K-driven proliferation in our 3D culture model; these results were published and reported in our 2012 Progress Report [4]. Despite these profound effects on primary tumor growth and oncogenic proliferation, the effects of p62/SQSTM1 on metastasis are unknown. Moreover, it remains unclear whether autophagy cargo adapters other than p62 impact metastasis. Thus, we evaluated whether the pulmonary

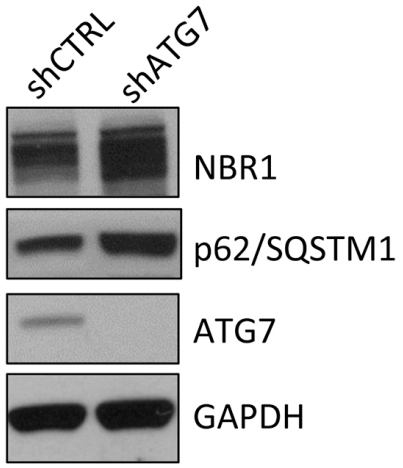


Fig 15: Metastatic ATG7 knockdown PyMT-R221 cells cultured from lungs of recipient mice exhibit increased levels of the autophagy cargo receptors, p62/SQSTM1 and NBR1.

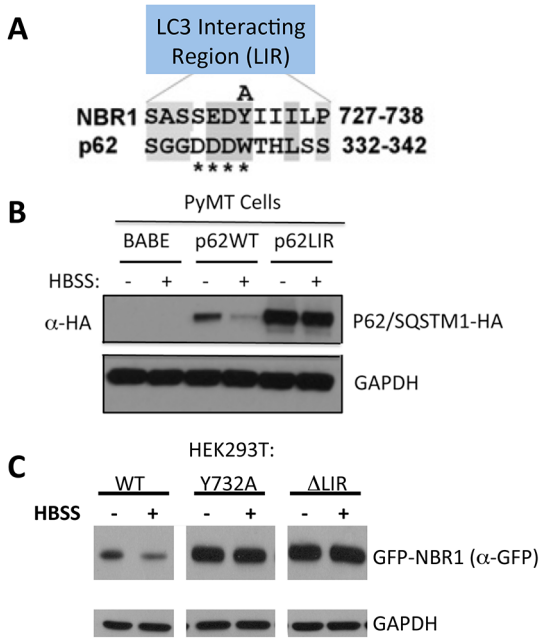


Fig 16: Generation of cells expressing autophagy-resistant mutants of p62 and NBR1. (A) LC3-interating region (LIR) in the autophagy cargo receptors p62 and NBR1. Point mutation of Y732 in NBR1 or W338 in p62 (to A) disrupts their ability to interact with LC3 and renders them autophagy resistant. (B) Introduction of p62WT and p62LIR (W338A) into PyMT-R221 cells. P62LIR are resistant to starvation-induced autophagy. (C) Expression of loss-of-function NBR1 LIR mutants in 293T cells; ΔLIR eliminates the 4 residues shown by asterisks in Fig 16A. Both mutant forms of NBR1 are resistant to starvation-induced autophagy.

metastasis from the autophagy-deficient models exhibited increased levels of autophagy cargo receptors. We confirmed an increase in p62/SQSTM1 and in a second autophagy cargo receptor, NBR1 (Neighbor of BRCA1) in metastatic cells culture from the lungs of mice transplanted with PyMT-shATG7 tumors (Fig. 15).

The striking accumulation of these autophagy receptors broaches the idea that they may contribute to the pro-metastatic phenotypes observed upon autophagy inhibition. To initially test this hypothesis, we will utilize a gain-of-function strategy and evaluate whether the enforced regulation of these receptors are sufficient to promote metastasis. Importantly, both p62/SQSTM1 and NBR1 require a specific motif in order to be degraded via autophagy, called the LC3/ATG8-interacting region (LIR) (Fig. 16A)[17]. As reported in our 2011 Annual Progress Report, sub-task 1a, we generated a mutant version of p62, called p62-LIR, containing a point mutation (W338A) in the LIR that rendered it resistant to degradation via autophagy [4]. We have successfully and stably expressed both p62 wild type and p62-LIR in PyMT cells and confirmed that the LIR mutant is resistant to autophagic degradation in response to starvation-induced autophagy (Fig 16B) and we are in the process of creating similar 4T1 cell derivatives. Furthermore, we have made the analogous mutation in the LIR of NBR1 (Y732A as well as a LIR deletion); upon expression in a 293T cells, we have confirmed that the LIR mutant is resistant to degradation via autophagy (Fig 16C). Using these stable expression vectors, over the upcoming years, we will generate PyMT cells containing NBR1 wild type and NBR1-LIR similar to our p62 lines shown in Fig. 16B. These cells will be utilized to evaluate whether the accumulation of either autophagy cargo receptor is sufficient to phenocopy the pro-metastatic phenotype that we have observed in autophagy-deficient cells.

Vascular changes in autophagy-deficient tumors: Because our results from sub-task 1b indicated a role for autophagy in the support of secretion, we began studies to test if the increase in metastasis may paradoxically arise from a defect in autophagy-dependent secretion of angiogenic factors. One of the most striking phenotypes we have uncovered in the primary tumors has been that 4T1-shATG7 tumors exhibit an alteration in the tumor vasculature. FITC-conjugated dextran injection studies revealed decreased extravascular fluorescence in 4T1-shAtg7 tumors, indicative of reduced vessel leakage. In further support, 4T1-shATG7 tumors exhibited decreased fibrinogen staining, a clotting factor deposited as a

provisional matrix and thus a surrogate marker for vascular leakage (Fig. 17). These results motivate the prediction that autophagy in 4T1 tumor cells results in the production of specific secreted factors that drive aggressive neo-vascularization, resulting in the characteristic leaky vessels found in angiogenic tumors. We hypothesize that disruption of this secretome in autophagy-deficient 4T1 tumors unexpectedly promotes metastatic colonization by normalizing the local vasculature at the metastatic site. We will further explore this hypothesis in the upcoming year.

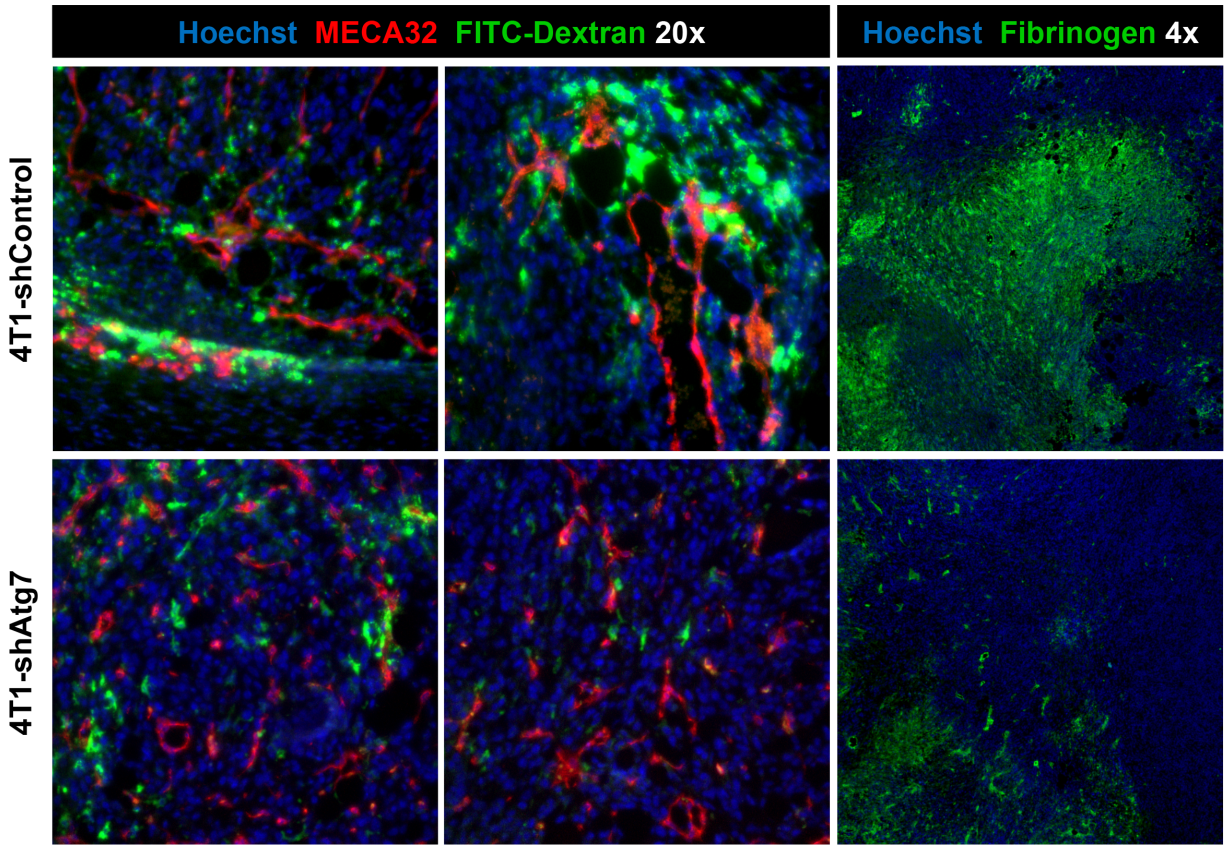


Fig. 17: Effects of 4T1 tumor cell autophagy deficiency on vascular permeability. FITC-Dextran (green) was injected into the tail veins of tumor-bearing mice and visualized by microscopy. Vessels were stained with antibodies against the endothelial marker MECA32 (red). Representative images show a decrease in extravascular FITC-dextran, suggesting of decreased vascular permeability, in Atg7 knockdown tumors compared to controls (left). This result is supported by fibrinogen IHC, a clotting factor that decreases in Atg7 knockdown tumors (right). Nuclei were counterstained with Hoechst.

Effects of autophagy inhibition on focal adhesion (FA) remodeling: A growing body of work suggests that increase focal adhesion-based signaling may serve a key determinant in the outgrowth of metastatic colonies. As a result, we evaluated whether autophagy inhibition impacted focal adhesions and observed that autophagy-deficient mammary cancer cells, including the PyMT-shATG7 cells, exhibit an increase in focal adhesion size; similar results were observed in HRasV12-transformed MCF10A cells, suggesting a general role for autophagy in the control of focal adhesions (Fig. 18A & B). Because FAs undergo cycles of turnover and remodeling in which they both assemble and disassemble in order for cells to migrate, these increases in FA size suggest defective turnover in autophagy-deficient cells [18,19]. To clarify whether autophagy inhibition leads to specific defects in FA remodeling, we generated control and ATG knockdown HRasV12 MCF10A cells expressing mCherry-paxillin, an established reporter useful for monitoring dynamic FA turnover in migrating cells via spinning disc confocal microscopy (Fig. 19A) [20]. We conducted real-time dynamic imaging studies in collaboration, which revealed that ATG knockdown led to significantly reduced FA disassembly and increased FA lifetime (Fig. 19B).

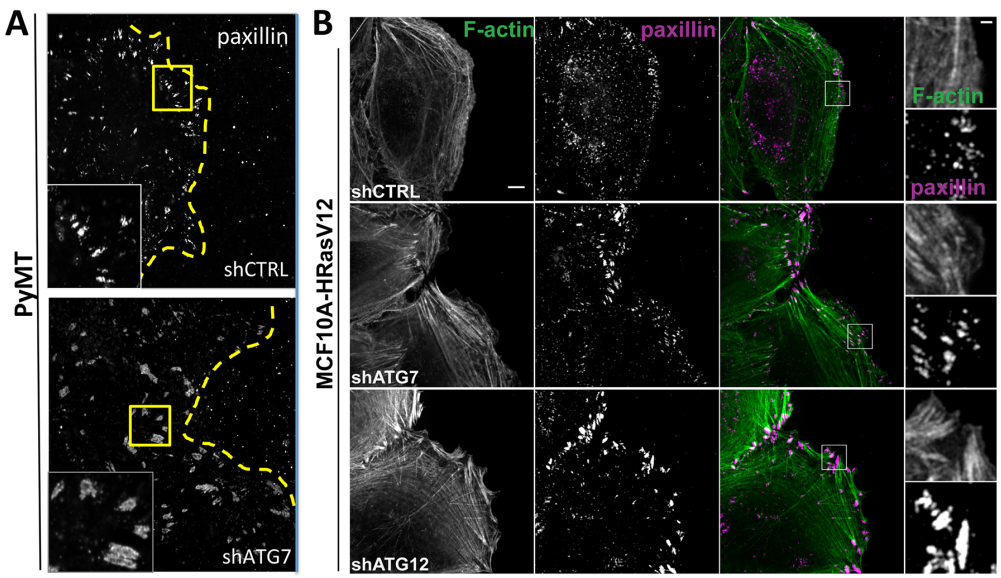


Fig 18: Increased focal adhesion size upon autophagy inhibition in PyMT-R221 (A) in HRAS^{V12} MCF10A cells (B) The indicated cell types from in vitro wounding assays were stained with the focal adhesion protein paxillin to detect focal adhesions. Boxed areas are magnified in the insets.

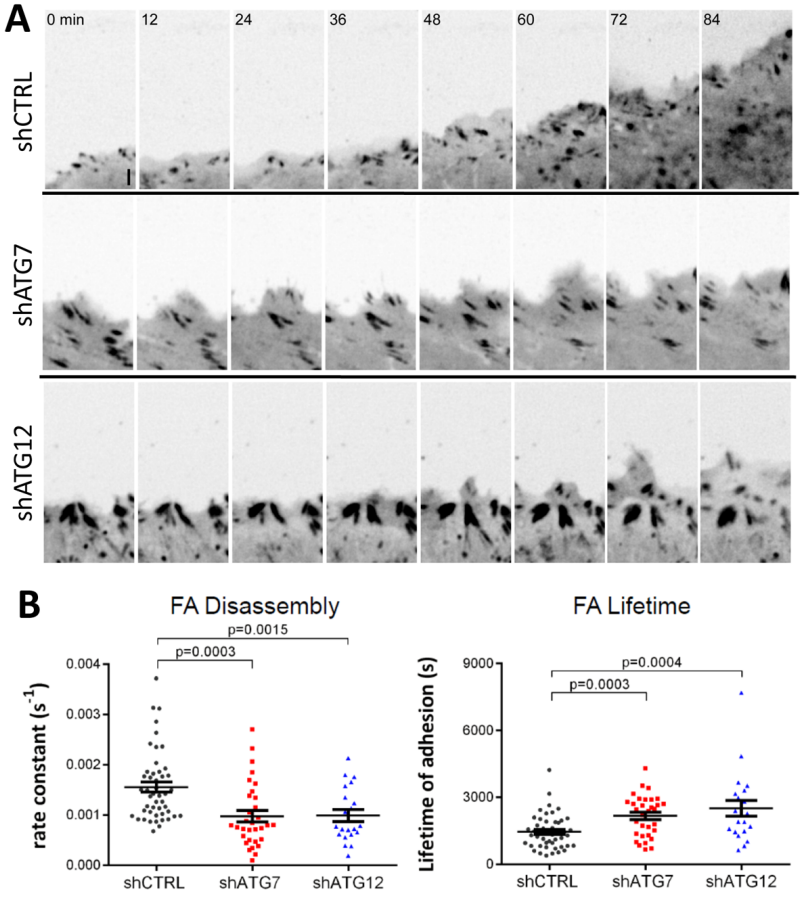


Fig 19: Dynamic imaging of focal adhesion turnover in HRAS^{V12} MCF10A cells. A) Representative still images of mCherry-paxillin labeled FAs over 84' time-lapse. B) Quantitative analysis of FA dynamics confirms impaired adhesion disassembly and increased FA lifetime in shATG7/12 cells.

FA-derived intracellular signals serve as key mediators of cell cycle progression and proliferation; among these signals, focal adhesion kinase (FAK) is thought to play a central role [21]. FAK activation has been demonstrated to be vital for disseminated tumor cells to proliferate and develop into overt macrometastasis [22-25]. Accordingly, we observe increased FAK activation (P-FAK^{Y397}) in ATG knockdown cells in vitro (Fig. 20). Over the upcoming year, we will test if aberrant activation of adhesion-based proliferative signals, namely FAK, promotes the metastatic colonization autophagy-deficient tumor cells. For these studies, we have been leveraging the 3D assays developed during year 1 in sub-task 1a. Recent work highlights the utility of this approach to model salient features of post-extravasation and early colonization behavior in vitro; briefly, cells are plated at low density (500 cells/well) using "Matrigel on top" 3D culture conditions, using protocols we developed for sub-task 1a and subject to phase morphological and focal adhesion analysis at 24h [25,26]. We conducted initial studies using autophagy-competent and autophagy-deficient PyMT-R221 cells; as shown in Fig. 21, control cells remain rounded upon initial replating while shAtg7 cells exhibit increased spreading onto the extracellular matrix. We will now employ these assays to test whether autophagy depletion elicits increased adhesion size and elongated adhesion plaques [26], as well as enhanced FAK (P-FAK^{Y397}) activation.

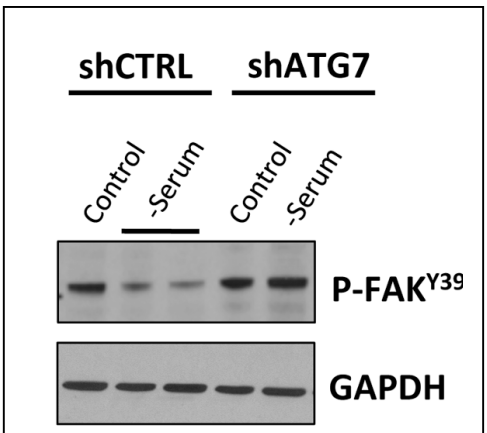


Fig 20: Increased P-FAK^{Y397} levels in shATG7 cells following 18h serum starvation (in media with 0.5% FBS). Control=full media with 10% FBS.

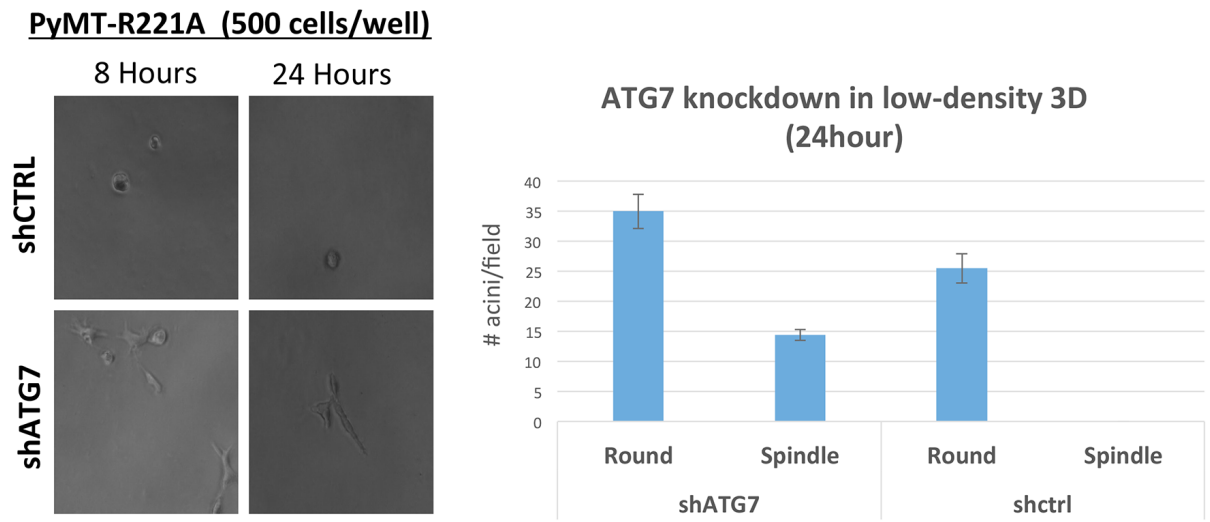


Fig 21: Low density 3D culture assay to evaluate early metastatic capacity in vitro. PyMT-R221 cells were seeded at low density (500 cells per well (1cm^2 surface area) on a 3D overlay ("Matrigel on top") assay. (A) Phase microscopy reveals that autophagy competent cells (shCTRL) appeared rounded, whereas shATG7 cells exhibit an elongated, spindled morphology and invasive protrusions. (B) Quantification of rounded versus spindled morphology at 24h following plating.

Altogether, the results from these three initial areas of investigation suggests that autophagy inhibition promotes the early steps of metastatic seeding via the control of adhesion; at the same time, the modulation of secreted factors and the activation of cell-autonomous signals secondary to the accumulation of autophagy cargo receptors may contribute to metastatic colonization at the foreign tissue site. We anticipate that further delineating these pro-metastatic activities will mechanistically inform how autophagy impacts tumor cell exit from dormancy and toward overt metastatic disease. Thus, during the

upcoming years, one of the important goals for both sub-tasks 2c and 2d will be to follow-up salient results gleaned from the PyMT and 4T1 models in the ongoing late recurrence studies using the transplanted mice from sub-task 2b.

d. If late onset metastasis does occur in autophagy deficient tumor cell recipients from subtasks 2b-c, isolate late onset macro-metastatic tumors and dormant tumor cells and obtain gene expression profiles. To obtain dormant cells from each cohort in subtask 2b, ten (10) tumor cell bearing mice from 2b will be euthanized at an intermediate time point (anticipated to occur at 6-9 months post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 37-48).

These studies have not been initiated.

e. If metastasis is significantly reduced or eliminated in autophagy deficient tumor cell recipients from subtasks 2b-c, determine the effects of antimalarial treatment on the onset of metastasis in mice transplanted with autophagy competent neoplastic breast cells, using appropriate transplantation models determined from subtask 1d and/or subtask 1i-j (Months 37-60).

These studies have not been initiated. Although our studies indicate that metastasis is enhanced, rather than reduced, in autophagy deficient tumor cell recipients, this sub-task is still important to pursue because our unexpected findings raise the possibility that anti-malarials such as hydroxychloroquine (HCQ) as pharmacological autophagy inhibitors may harbor long-term risks by enhancing metastasis in breast cancer patients. These studies in the upcoming years will illuminate this critical issue.

Task 3: Determine if dietary restriction or mammalian target of rapamycin (mTOR) inhibition can prevent the expansion of dormant cells into overt metastases.

a. Isolate neoplastic epithelium from female transgenic mice generated in subtask 1f, transduce with Cre recombinase, and transplant into cleared mammary fad pads of syngeneic C57B/6 host recipient animals. For this subtask, ten (10) donor mice will be utilized for epithelial isolation. Twenty-five (25) host recipient animals per cohort necessary for subtasks 3b and 3d will be utilized for fat pad transplantation (Months 37-60).

These studies have not been initiated.

b. Determine the effects of dietary restriction (DR), defined as a 40% reduction in caloric intake, on the onset of overt and latent metastasis for recipient mice generated in subtask 3a. We anticipate that twenty-five (25) mice each from two (2) cohorts (cohort 1: subject to DR; cohort 2: controls not subject to DR) will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 49-60).

These studies have not been initiated.

c. From mice in subtask 3b, isolate late onset macro-metastases (if they occur) and dormant tumor cells and obtain gene expression, biosynthetic, and metabolic profiles. To obtain dormant cells from each cohort in subtask 3b, ten (10) tumor cell bearing mice from 3b will be euthanized at an intermediate time point (anticipated to occur at 6-9 month post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 49-60).

These studies have not been initiated.

d. Determine the effects of mTOR inhibition on the onset of overt and latent metastasis for recipient mice generated in subtask 3a. Twenty-five (25) mice per cohort will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 37-60).

These studies have not been initiated.

e. From mice in subtask 3d, isolate late onset macro-metastases (if they occur) and dormant tumor cells and obtain gene expression, biosynthetic, and metabolic profiles. To obtain dormant cells from each cohort in subtask 3b, ten (10) tumor cell bearing mice from 3d will be euthanized at an intermediate time point (anticipated to occur at 6-9 month post fat pad transplantation but prior to overt metastatic disease), cancer cells isolated from host tissues, and if required, expanded ex vivo (Months 49-60).

These studies have not been initiated.

III. KEY RESEARCH ACCOMPLISHMENTS:

Year 1 (Months 1-12):

1. We optimized an overlay ("on-top") three-dimensional organotypic culture system on laminin-rich reconstituted basement membrane suitable for the culture of oncogene-expressing mammary epithelial cells and human breast cancer cells.
2. We uncovered that the anti-malarial chloroquine (CQ) inhibits quiescent behavior in response to mTOR pathway inhibition in mammary epithelial cells expressing a tumor-derived, activating mutation in phosphatidylinositol 3-kinase (PI3K), H1047R. These data support a model that autophagy inhibition may paradoxically promote the active growth of quiescent cells via the MAPK pathway secondary to the accumulation of the autophagy substrate, p62/SQSTM1. **Published in Chen et. al. Oncogene, 2012.**
3. Based on the 3D proliferation rates of 6 human breast cancer cell lines tested during the first year, we identified two ER+ cell lines MCF7 and T47D as lead candidates for further evaluation as potential models for in vivo dormant behavior.
4. We discovered that the antimalarials chloroquine (CQ) and quinacrine (Qu) are able to inhibit the 3D growth and proliferation of MCF7 cells at levels comparable to tamoxifen (OHT).
5. We optimized protocols for the lentiviral-mediated delivery of reporter proteins and the drug resistance markers to breast cancer cells.
6. We optimized protocols for the successful isolation of mammary organoids from normal and PyMT tumor-bearing mice.
7. We developed protocols to deliver adenoviral Cre in order to excise the ATG12 conditional allele (*atg12f/f*) ex vivo from organoids derived from normal mammary gland and PyMT tumors. Upon reintroduction of cells into the mammary fad pads of recipient mice, we confirmed both ATG12 deletion and defective autophagy in the resultant tumors arising from ATG12 deleted tumor cells.
8. We have generated Polyoma Middle T (PyMT), ROSA26-LSL-RFP, and the ATG conditional mouse strains (*atg12f* and *atg5f*) in a pure C57B/6 genetic background.

Year 2 (Months 12-24):

9. We have extended our three-dimensional culture studies of PI3K-H1047R and evaluated the effects of ATG depletion on the morphogenesis of breast epithelial cells transformed with oncogenic Ras.

Autophagy inhibition does not impact proliferation in this model, indicating that the effects of autophagy on quiescent behavior in 3D culture are oncogene-dependent.

10. In Ras-transformed breast cancer cells, we have found that the depletion of autophagy-related genes suppresses invasion in three-dimensional culture and reduces pulmonary metastases *in vivo*.
11. Based on conditioned media experiments in 3D culture, we have found that autophagy-deficient Ras cells, fail to secrete pro-invasive factors. We have also discovered that reduced autophagy diminishes the secretion of the pro-migratory cytokine, interleukin-6, which is necessary and sufficient to restore invasion of autophagy-deficient cells.
12. We have completed the interbreeding to generate compound transgenic mice containing Polyoma middle T (PyMT), ATG conditional alleles, and ROSA26-LSL-RFP in a C57B/6 genetic background. We have harvested primary cells from tumor bearing mice, validated the ability to genetically delete ATGs, as well as reintroduce these cells into a syngeneic C57B/6 host.
13. We have confirmed micro-metastatic lesions upon tail vein injection of PyMT cells and the presence of solitary tumor cells within lung tissue of recipient mice at an early time point of a spontaneous metastasis assay, suggesting that this approach will be useful to study recurrence over extended periods of time.
14. We have created a bank of frozen primary PyMT-derived tumor cells in which we can ablate autophagy (via Cre deletion of *atg12* or *atg5*) as well as carry out transplantation studies in the upcoming years.
15. We have created an immortalized cell line from a PyMT *atg12f/f* tumor isolated in our laboratory, which will be valuable for dissecting the mechanisms responsible for the phenotypes from experiments using primary tumor-derived tissue in years 3-5.
16. Using stable RNAi-mediated depletion of ATG7 in a PyMT cell line, we have found that autophagy inhibition accelerates the outgrowth of overt metastasis in the lungs of syngeneic recipient mice. Overall, this result provides in vivo corroboration for our 3D studies of PI3K-H1047R during year 1 and suggests that increasing autophagy in the host animal may be useful in preventing late recurrent disease.

Year 3 (Months 25-36):

17. We uncovered that autophagy promotes the transcription and production of the secreted factors, WNT5A and MMP2, in breast epithelial cells transformed with oncogenic Ras. Overall, these results support that autophagy in promoting cancer cell invasion via the coordinate production of multiple secreted factors in addition to IL6, which may impact late recurrent disease and metastatic outgrowth in vivo. **Published in Lock et. al., Cancer Discovery, 2014.**
18. We expanded our bank of frozen primary PyMT-derived tumor cells in which we can ablate autophagy (via Cre deletion of *atg12* or *atg5*) as well as continue to carry out transplantation studies in the upcoming years.
19. We initiated transplantation experiments using the primary PyMT tumors we had created in previous years. These mice will be analyzed for the onset of pulmonary macro-metastasis during the upcoming year. Analysis of an intermediate time point confirmed the persistence of solitary tumor cells within lung tissue of recipient mice from a spontaneous metastasis assay and broached the possibility of late metastatic recurrence over extended periods of time.
20. We generated compound transgenic mice containing a tamoxifen-inducible Cre recombinase (CAG-Cre^{ERT}) along with Polyoma middle T (PyMT), *atg12f/f*, and ROSA26-LSL-RFP, all in a C57B/6 genetic background. Upon harvesting tumors from these mice, we have confirmed efficient ATG12 deletion ex vivo upon treatment with 4-hydroxytamoxifen (4OHT) as well as confirmed the complete ablation of autophagy. This new model will allow for the temporal specific deletion of ATGs in vivo.
21. Using stable RNAi-mediated depletion of ATG7 and ATG12 in the 4T1 cell line, we corroborated that autophagy inhibition accelerates the outgrowth of overt metastasis in the lungs of syngeneic recipient mice. Overall, this result validates our year 2 results from the PyMT-R221 model using an immune competent transplantation model and suggests that increasing autophagy in the host animal may be

useful in preventing late recurrent disease. Over the upcoming years, a major goal will be to evaluate this new concept using the primary slow progression PyMT models.

22. We have uncovered potential mechanisms for the pro-metastatic phenotypes observed in autophagy deficient cells. These include: 1) activation of pro-metastatic pathways due to the accumulation of the autophagy cargo receptors p62/SQSTM1 and NBR1; 2) changes in angiogenesis due to modulation of secreted angiogenic factors; and 3) autophagy-dependent changes in focal adhesion remodeling that may impact metastatic seeding or colonization.
23. Using the 3D overlay model developed in year 1, sub-task 1a, we find that autophagy-deficient PyMT-R221 cells exhibit enhanced spreading during low density re-seeding, providing additional evidence that autophagy may restrict metastasis by impeding early seeding and colonization events in vivo.

IV. REPORTABLE OUTCOMES:

Publications

Months 1-12 (provided as Appendix material in 2012 Progress Report):

1. **J. Debnath** (2011). The Multifaceted Roles of Autophagy in Breast Cancer. *J. Mamm. Gland Biol. Neopl.* 16 (3): 173-87. PMID: 21779879 PMCID: PMC3170851.

2. N. Chen, Eritja N., Lock R., **Debnath J.** (2012). Autophagy Restricts Proliferation Driven By Oncogenic Phosphatidylinositol 3-Kinase in Three-Dimensional Culture. *Oncogene.* doi: 10.1038/onc.2012.277. 2012 Jul 9. Epub ahead of print. PMID: 22777351 PMCID: PMC3470740.

Months 12-24 (provided as Appendix material in 2013 Progress Report):

1. Murrow, L, **Debnath, J.** (2013) Autophagy as a Stress Response and Quality Control Mechanism: Implications For Cell Injury and Human Disease. *Annu Rev Pathol.,* 2013, 8:105-137. PMID: 23072311. PMCID: PMC3971121.

2. M.S. Sosa, P. Bragado, **J. Debnath*,** J. A. Aguirre-Ghiso*. Regulation of Tumor Cell Dormancy By Tissue Microenvironments and Autophagy (2013). Adv. Exp. Med. Biol, 734: 73-89. PMID: 23143976. PMCID: PMC3651695. ***Co-senior author.**

3. N. Chen, **Debnath J.** (2013). IkB Kinase (IKK) Triggers Detachment-Induced Autophagy In Mammary Epithelial Cells Independently of the PI3K/AKT/MTORC1 Pathway. *Autophagy.* 9(8): 1214-27. PMCID: PMC3748193

Months 25-36 (reported in Appendix B):

1. R. Lock, Kenific, C.M., Leidal, A.M., Salas, E., and **Debnath, J.** (2014) Autophagy dependent production of secreted factors facilitates RAS-driven invasion. *Cancer Discovery*. 4(4): 466-79. PMCID: PMC3980002.

2. Goldsmith, J, Levine, B., and **Debnath, J.** (2014). Autophagy and cancer metabolism. *Methods Enzymol.* 542: 25-57. PMID: 24862259.

3. Leidal, A.M. and **Debnath, J.** (2014). "Doubling down" on the autophagy pathway to suppress tumor growth. *Genes and Development.* 2014; 28(11): 1137-9. PMID: 24888584.

Presentations (Months 25-36):

International and National Conferences:

2014 Invited Speaker, Gordon Research Conference on Autophagy, Il Ciocco, Italy
2014 Invited Speaker, Educational Session on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, San Diego, CA
2014 Invited Speaker and Symposium Chair, Experimental Biology 2014, ASIP Symposium on Cancer Pathobiology, San Diego, CA
2014 Invited Speaker, Educational Session on Signaling Pathways and Therapeutics, American Society of Clinical Oncology Annual Meeting, Chicago, IL
2014 Invited Speaker, National Breast Cancer Coalition Artemis Meeting on Tumor Dormancy, Calistoga, CA
2014 Invited Speaker, National Breast Cancer Coalition Leadership Summit, Alexandria, VA
2014 Invited Speaker, Samuel Waxman Cancer Research Foundation Meeting, New York, NY
2014 Invited Speaker, Keystone Symposium on Autophagy and Disease, Austin, TX

Invited Lectures and Seminars:

2013 Invited Seminar, Amgen Department of Oncology, San Francisco, CA
2014 Invited Seminar, Institut Pasteur, Paris, France
2014 Invited Seminar, Program in Cancer Biology, University of Hawaii Cancer Center, Honolulu, HI

Patents and Licenses (Months 1-36):

None.

Degrees Obtained (Months (1-36):

None.

Reagent Development (Months 1-36):

Generation of MMTV-PyMT mice on the C57B/6 strain background.
Generation of *atg12f*, LSL-RFP mice on the C57B/6 strain background.
Generation of *atg5f* mice on the C57B/6 strain background.
Generation of *atg5f*, LSL-RFP mice on the C57B/6 strain background.
Generation of MMTV-PyMT, *atg12f*, LSL-RFP mice on the C57B/6 strain background.
Generation of mammary carcinoma cell line derived from an MMTV-PyMT, *atg12f*, β-actin CFP, ROSA26-LSL-RFP host tumor suitable for transplantation into C57B/6 syngeneic host animals.
Generation of MMTV-PyMT, atg12f, LSL-RFP, CAG-Cre^{ERT} mouse on a C57B/6 strain background.

Funding Applied For Based On Work Supported By Era of Hope (Months 24-36):

1. Debnath, Jayanta (PI): NIH R01 CA188404
Status: AWARDED (9/1/2014-8/30/2019)

2. Debnath, Jayanta (PI): NIH R01 CA126792
Status: PENDING (2/1/2015-1/31/2020)

Employment and Research Opportunities:

None.

V. CONCLUSION:

The biological processes that govern the critical steps in late recurrent disease in breast cancer remain largely unclear [2]. This project focuses on how the fundamental stress pathway autophagy impacts quiescent versus proliferative behavior exhibited by breast cancer cells. To meet this objective, we created both 3D organotypic culture models to assess quiescent behavior displayed by breast cancer cells as well as in vivo mouse models to recapitulate late recurrent breast cancer.

Our initial studies using a three-dimensional organotypic culture model to investigate oncogenic PI3K-expressing mammary epithelial cells and human breast cancer cell lines previously uncovered that autophagy restricts proliferation and maintains a quiescent state [4]. To follow-up these findings, over the past year, we evaluated the role of autophagy during metastatic progression using PyMT and 4T1 mouse tumor cell lines transplanted into syngeneic immune competent hosts; in both of these models, we observed that autophagy inhibition promotes, rather than impedes the metastasis of PyMT cells in vivo, thereby corroborating our in vitro findings. These unexpected findings raise the possibility that pharmacological autophagy inhibitors like hydroxychloroquine (HCQ) may harbor long-term risks by enhancing metastasis in certain cancer patients. Accordingly, an important goal of this proposal is to more precisely define the stage at which autophagy suppresses mammary cancer metastasis in vivo and to dissect the mechanisms by which autophagy deficiency promotes the metastatic phenotype. During the past year, we have found that autophagy inhibition potentially promotes the early steps of metastatic seeding via the control of adhesion; at the same time, we have observed the accumulation of two autophagy cargo receptors—p62 and NBR1. During the upcoming year, we will assess whether the activation of cell-autonomous signals secondary to the accumulation of these two autophagy cargo receptors may contribute to metastatic colonization at the foreign tissue site.

At the same time, we also uncovered using our 3D models that autophagy promotes invasive behavior and alters epithelial differentiation and secretion in mammary epithelial cells expressing oncogenic Ras. In year 2, we identified interleukin-6 as one critical secreted factor that was necessary and sufficient to restore invasion of autophagy-deficient cells. Over the past year, we extended these results and found that, in addition to IL6, autophagy promotes the transcription and production of the secreted factors, WNT5A and MMP2, in breast epithelial cells transformed with oncogenic Ras. Overall, these results support that autophagy in promoting cancer cell invasion via the coordinate production of multiple secreted factors, which may impact late recurrent disease and metastatic outgrowth in vivo. These findings were published over the past year [5]. Although these findings would suggest that autophagy suppresses invasion and metastasis, it is important to recognize that these alterations in autophagy-dependent secretion also result in enhanced epithelial differentiation in autophagy deficient cells grown in 3D culture [5]. Because the process of mesenchymal-to-epithelial transition has been shown to support active metastatic colonization [27], one can alternatively speculate that the enhanced metastatic outgrowth we have observed in vivo may partly arise from enhance epithelial differentiation, as we have observed upon autophagy inhibition in cells with Ras/MAPK pathway activation. At the same time, impaired secretion in autophagy-deficient tumor cells may alter the surrounding microenviroment, resulting in enhanced metastasis. In potential support of this concept, we have observed changes in the vasculature of autophagy-deficient tumors, and accordingly, we will evaluate whether and how such changes contribute to the pro-metastatic phenotypes observed in autophagy-deficient tumor cells.

Overall, our findings to date suggest that autophagy may promote late recurrent disease by promoting the exit of dormant tumor cells from quiescent states to produce overt metastatic disease. To address this question, we are utilizing the MMTV-PyMT mammary cancer progression model in a pure C57B/6 strain. We will exploit the delayed kinetics of PyMT tumor progression in C57B/6 mice to more effectively model late recurrence and

assess the effects of autophagy on dormant tumor cell behavior [28]. We have: 1) created compound transgenic mice containing MMTV-PyMT and ATG conditional alleles on the C57B/6 strain background, including a strain containing a tamoxifen-inducible Cre (CAG-Cre^{ERT}) that allow for both temporal control of autophagy inhibition; 2) isolated tumors from these animals and confirmed that autophagy can be conditionally ablated ex vivo or in vivo, and 3) created a bank of frozen primary PyMT-derived tumor cells in which we can ablate autophagy. Upon transplantation of these primary tumor cells into the mammary fat pad of syngeneic recipient mice, we have confirmed the presence of the presence of solitary tumor cells within lung tissue of recipient mice point, suggesting that this approach will be useful to study late recurrence and metastasis.

Impact: Late recurrent breast cancer is highly resistant to available treatments and commonly metastatic; hence, it is a principal cause of lethality in breast cancer patients. By generating robust in vivo systems for late recurrent disease, we will address this essential unmet need in breast cancer research. To eliminate late recurrent breast cancer, one potential strategy may be to impede their ability to “reawaken” and develop into lethal macro-metastases by promoting autophagy. Our studies using two established immune-competent models of breast cancer metastasis suggest that autophagy impedes rather than promotes the active outgrowth and early metastatic colonization. If we corroborate a similar functional requirement for autophagy in the mediating the outgrowth of quiescent cells during late recurrent disease, we will seek to modulate this fundamental process for therapeutic benefit in the prevention of lethal metastasis.

VI: BIBLIOGRAPHY:

1. Aguirre-Ghiso JA: **Models, mechanisms and clinical evidence for cancer dormancy**. *Nat Rev Cancer* 2007, **7**:834-846.
2. Debnath J: **The multifaceted roles of autophagy in tumors-implications for breast cancer**. *J Mammary Gland Biol Neoplasia* 2011, **16**:173-187.
3. Sosa MS, Bragado P, Debnath J, Aguirre-Ghiso JA: **Regulation of tumor cell dormancy by tissue microenvironments and autophagy**. *Adv Exp Med Biol* 2013, **734**:73-89.
4. Chen N, Eritja N, Lock R, Debnath J: **Autophagy restricts proliferation driven by oncogenic phosphatidylinositol 3-kinase in three-dimensional culture**. *Oncogene* 2012.
5. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J: **Autophagy-Dependent Production of Secreted Factors Facilitates Oncogenic RAS-Driven Invasion**. *Cancer Discov* 2014, **4**:466-479.
6. Fantozzi A, Christofori G: **Mouse models of breast cancer metastasis**. *Breast Cancer Res* 2006, **8**:212.
7. Chatterjee S, Seifried L, Feigin ME, Gibbons DL, Scuoppo C, Lin W, Rizvi ZH, Lind E, Dissanayake D, Kurie J, et al.: **Dysregulation of cell polarity proteins synergize with oncogenes or the microenvironment to induce invasive behavior in epithelial cells**. *PLoS One* 2012, **7**:e34343.
8. Debnath J, Brugge JS: **Modelling glandular epithelial cancers in three-dimensional cultures**. *Nat Rev Cancer* 2005, **5**:675-688.
9. Hayashi S, McMahon AP: **Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse**. *Dev Biol* 2002, **244**:305-318.
10. Luche H, Weber O, Nageswara Rao T, Blum C, Fehling HJ: **Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies**. *Eur J Immunol* 2007, **37**:43-53.
11. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T: **p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death**. *J Cell Biol* 2005, **171**:603-614.
12. Valastyan S, Weinberg RA: **Tumor metastasis: molecular insights and evolving paradigms**. *Cell* 2011, **147**:275-292.
13. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, et al.: **Autophagy suppresses tumorigenesis through elimination of p62**. *Cell* 2009, **137**:1062-1075.

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15. Komatsu M: **Potential role of p62 in tumor development**. *Autophagy* 2011, **7**:1088-1090.
16. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N: **Autophagy-deficient mice develop multiple liver tumors**. *Genes Dev* 2011, **25**:795-800.
17. Birgisdottir AB, Lamark T, Johansen T: **The LIR motif - crucial for selective autophagy**. *J Cell Sci* 2013, **126**:3237-3247.
18. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF: **FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly**. *Nat Cell Biol* 2004, **6**:154-161.
19. Webb DJ, Parsons JT, Horwitz AF: **Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again**. *Nat Cell Biol* 2002, **4**:E97-100.
20. Stehbens S, Pemble H, Murrow L, Wittmann T: **Imaging intracellular protein dynamics by spinning disk confocal microscopy**. *Methods Enzymol* 2012, **504**:293-313.
21. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC: **The role of focal-adhesion kinase in cancer - a new therapeutic opportunity**. *Nat Rev Cancer* 2005, **5**:505-515.
22. Benlimame N, He Q, Jie S, Xiao D, Xu YJ, Loignon M, Schlaepfer DD, Alaoui-Jamali MA: **FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion**. *J Cell Biol* 2005, **171**:505-516.
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24. Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG: **Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling**. *J Clin Invest* 2009, **119**:252-266.
25. Shibue T, Weinberg RA: **Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs**. *Proc Natl Acad Sci U S A* 2009, **106**:10290-10295.
26. Shibue T, Brooks MW, Inan MF, Reinhardt F, Weinberg RA: **The outgrowth of micrometastases is enabled by the formation of filopodium-like protrusions**. *Cancer Discov* 2012, **2**:706-721.
27. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J: **Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis**. *Cancer Cell* 2012, **22**:725-736.
28. Lifsted T, Le Voyer T, Williams M, Muller W, Klein-Szanto A, Buetow KH, Hunter KW: **Identification of inbred mouse strains harboring genetic modifiers of mammary tumor age of onset and metastatic progression**. *Int J Cancer* 1998, **77**:640-644.

VII: APPENDICES:

A. Curriculum Vitae of Jayanta Debnath.
B. Collected publications from Months 25-36.

University of California, San Francisco

CURRICULUM VITAE

Name: Jayanta Debnath, MD

Position: Associate Professor, Step 3
Pathology
School of Medicine

Address: Box 0502
Core Campus, HSW, 450B
University of California, San Francisco
San Francisco, CA 94143

email: jayanta.debnath@ucsf.edu

EDUCATION

1988 - 1992	Georgia Institute of Technology	B.S.	Highest Honors, Chemistry
1992 - 1998	Harvard Medical School	M.D.	Magna cum laude
1995 - 1997	National Cancer Institute, NIH	HHMI Research Scholar	Harold Varmus Lab
1998 - 1999	Brigham and Women's Hospital	Intern	Pathology
1999 - 2000	Brigham and Women's Hospital	Resident	Pathology
2000 - 2003	Brigham and Women's Hospital	Fellow	Pathology
2000 - 2005	Harvard Medical School	Postdoctoral Fellow	Cell Biology (Joan Brugge Lab)

LICENSES, CERTIFICATION

2003	Massachusetts Medical License Board of Registration in Medicine (expired 2006)
2003	American Board of Pathology, Board Certification in Anatomic Pathology
2005	California Medical Board, Physician and Surgeon

PRINCIPAL POSITIONS HELD

2003 - 2005	Harvard Medical School	Instructor	Cell Biology

2005 - 2011	University of California, San Francisco	Assistant Professor	Pathology
2011 - present	University of California, San Francisco	Associate Professor	Pathology

OTHER POSITIONS HELD CONCURRENTLY

2005 - present	UCSF Medical Center	Staff Pathologist

HONORS AND AWARDS

1988	President's Scholar (Full academic scholarship)	Georgia Institute of Technology
1989	Outstanding freshman, sophomore, junior, and senior awards, School of Chemistry	Georgia Institute of Technology
1991	Undergraduate Research Fellowship in Chemistry	National Science Foundation
1992	Phi Kappa Phi Scholarship Cup (Valedictorian)	Georgia Institute of Technology
1995	NIH-HHMI Research Scholar	Howard Hughes Medical Institute
1997	Research Award for Continued Medical Studies	Howard Hughes Medical Institute
1998	Honors in a Special Field, magna cum laude	Harvard Medical School
1998	Soma Weiss Medical Student Research Day Speaker Award	Harvard Medical School
2000	HHMI Postdoctoral Fellowship for Physicians	Howard Hughes Medical Institute
2003	Mentored Clinical Scientist Development Award (KO8)	National Cancer Institute
2003	Pfizer Scholar-In-Training Award	American Association of Cancer Research
2006	Culpeper Scholar Award in the Medical Sciences	Partnership for Cures
2006	AACR Genentech BioOncology Career Award	AACR
2006	HHMI Physician-Scientist Early Career Award	Howard Hughes Medical Institute
2007	Stewart Family Trust Award	UCSF Cancer Center
2009	Aspen Cancer Conference Fellow	Aspen Cancer Conference
2011	Era of Hope Scholar Award	DOD Breast Cancer Research Program
2013	Elected to Membership in ASCI	American Society for Clinical Investigation

2013	Waxman Award	Samuel Waxman Cancer Research Foundation

KEYWORDS/AREAS OF INTEREST

autophagy, apoptosis, oncogenes, breast cancer, three-dimensional culture, extracellular matrix, integrins, ubiquitin-like molecules

PROFESSIONAL ACTIVITIES

CLINICAL

2005-present, Attending Pathologist, Department of Pathology Autopsy Service, UCSF Medical Center, San Francisco, CA

SUMMARY OF CLINICAL ACTIVITIES

I serve as an attending in the autopsy service at Moffitt Hospital at UCSF (6 weeks per year).

PROFESSIONAL ORGANIZATIONS

Memberships

2000 - present	American Society for Cell Biology
2004 - 2005	American Society of Investigative Pathology
2012 - present	American Society of Investigative Pathology
2006 - present	American Association of Cancer Research
2013 - present	American Society of Clinical Investigation (Elected)

Service to Professional Organizations

2004 -	American Society for Cell Biology	Participant, Capitol Hill Day (Advocacy for NIH budget)
2006 -	WICB Career Luncheon, American Society for Cell Biology	Discussion Leader
2009 -	Georgia Institute of Technology College of Science Advisory Board	Advisor
2009 -	2010 AACR Annual Meeting Program Committee (Cell Growth Signaling Pathways Subsection), American Association of Cancer Research	Subcommittee Member
2010 -	UCSF Diller Cancer Center Bay Area Workshop, "Cancer Cell Growth and Metabolism", UCSF Diller Family Comprehensive Cancer Center	Co-organizer (Conference Chair) with Davide Ruggero, UCSF
2010 -	National Cancer Institute (NIH) Workshop on Autophagy and Cancer	Co-chair with Eileen White, CINJ
2011 -	6th Era of Hope Conference, Orlando, FL, DOD	Technical Program

	Breast Cancer Research Program	Committee (Abstract Placement For Meeting)
2011 -	2011 ASCB Annual Meeting Special Session on Extracellular Matrix Regulation of Programmed Cell Death, Denver, CO	Co-organizer (with Mike Overholtzer, MSKCC)
2011 -	2012 AACR Annual Meeting Program Committee (Cell Death Subsection) American Association of Cancer Research	Subcommittee Member
2013 -	2014 AACR Annual Meeting Program Committee (Cell Death Subsection) American Association of Cancer Research	Subcommittee Member
2014 -	Experimental Biology 2014, ASIP Symposium on Cancer Pathobiology, San Diego, CA	Symposium Chair
2015 -	2015 Keystone Symposium on Autophagy	Co-organizer (with Eric Baehrecke, U. Mass.)

SERVICE TO PROFESSIONAL PUBLICATIONS

2008 - present Ad hoc referee for approximately 60 manuscripts per year. Since 2008, journals include:

- American Journal of Pathology, 1 paper
- Apoptosis, 1 paper
- Autophagy, 45 papers
- BBA Cancer Reviews, 1 paper
- BMC Cell Biology, 1 paper
- Breast Cancer Research, 1 paper
- Cancer Cell, 2 papers
- Cancer Discovery, 6 papers
- Cancer Research, 17 papers
- Chronobiology International, 1 paper
- Clinical Cancer Research, 3 papers
- Cell, 5 papers
- Cell Death and Differentiation, 6 papers
- Cell Metabolism, 2 papers
- Cell Research, 1 paper
- Current Biology, 2 papers
- Developmental Cell, 2 papers
- Disease Models and Mechanisms, 2 papers
- EMBO Journal, 1 paper

- EMBO Reports, 1 paper
- Experimental and Clinical Metastasis, 1 paper
- Experimental Cell Research, 1 paper
- Genes and Development, 5 papers
- Human Molecular Genetics, 2 papers
- Journal of Cell Biology, 4 papers
- Journal of Cell Science, 6 papers
- Journal of Clinical Investigation, 4 papers
- Mitochondrion, 1 paper
- Molecular Biology of the Cell, 13 papers
- Molecular Cancer Therapeutics, 2 papers
- Molecular and Cellular Biology, 12 papers
- Molecular Cell, 15 papers
- Molecular Therapy, 1 paper
- Nature, 7 papers
- Nature Cell Biology, 6 papers
- Nature Chemical Biology, 1 paper
- Nature Communications, 2 paper
- Nature Reviews Cancer, 2 papers
- Nature Reviews Clinical Oncology, 1 paper
- Nature Structural and Molecular Biology, 5 papers
- Oncogene, 31 papers
- PNAS, 5 papers
- PLOS Medicine, 1 paper
- PLOS One, 3 papers
- Radiation Research, 2 papers
- Science, 2 papers
- Science Signaling, 2 papers
- Science Translational Medicine, 3 papers
- Trends in Cell Biology, 1 paper

2010 - 2011 Editorial Board, Autophagy

2011 - present Associate Editor, Autophagy (63 papers)

2014 - Associate Editor, Molecular and Cellular Oncology (new journal starting on Oct 2014)

INVITED PRESENTATIONS

INTERNATIONAL

1997 Invited Lecture, International Meeting on Cytoplasmic Tyrosine Kinases, Stockholm, Sweden

2001 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium of Programmed Cell Death, Washington, D.C.

2005 Session Chair, Autophagy and Cell Death, Gordon Research Conference on Autophagy, Il Ciocco, Italy

2006 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Programmed Cell Death, San Diego, CA

2007 Invited Lecture and Session Chair, ASCB/ECI Engineering Cell Biology Meeting, Cambridge, MA

2008 Invited Lecture, Autophagy in Cell Death and Aging Session, Gordon Research Conference on Autophagy, Ventura, CA

2009 Invited Speaker, American Society of Cell Biology National Meeting, Minisymposium on Autophagy and Lysosomes, San Diego, CA

2009 Session Co-chair, Minisymposium on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, Denver, CO

2010 Invited Lecture, Stanley Korsmeyer Symposium on Autophagy and Apoptosis, American Association of Cancer Research Annual Meeting, Washington, D.C.

2010 Invited Lecture and Session Chairperson, Workshop on 3D Cultures, American Association of Cancer Research Annual Meeting, Washington, D.C.

2010 Invited Speaker, Selective Autophagy Session, Gordon Research Conference on Autophagy, Il Ciocco, Italy.

2011 Co-chair and speaker, American Society of Cell Biology National Meeting Special Session on "ECM Regulation of Programmed Cell Death," Denver, CO

2011 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Ubiquitin Related Proteins, Denver, CO

2011 Invited Speaker and Session Chair, Zing Conference on Autophagy, Mayan Riviera, Mexico.

2012 Invited Panelist, Session on “Autophagy and Disease” Gordon Research Conference on Autophagy, Ventura, CA.

2012 Invited Speaker, Experimental Biology 2012, ASIP

Session on Autophagy, San Diego, CA

2012 Invited Speaker, 6th International Symposium on Autophagy, Okinawa, Japan

2012 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Cancer Cell Biology, San Francisco, CA

2014 Invited Speaker, Gordon Research Conference on Autophagy, Il Ciocco, Italy

2014 Invited Speaker, Educational Session on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, San Diego, CA

2014 Invited Speaker and Symposium Chair, Experimental Biology 2014, ASIP Symposium on Cancer Pathobiology, San Diego, CA

2014 Invited Speaker, Educational Session on Signaling Pathways and Therapeutics, American Society of Clinical Oncology Annual Meeting, Chicago, IL

2015 Invited Speaker, Forum on Autophagy and Cancer, American Association of Cancer Research Annual Meeting, Philadelphia, PA

2015 Invited Speaker, Gordon Conference on Mammary Gland Biology, Mount Snow, VT

NATIONAL

2001 Invited Lecture, National Cancer Institute Workshop on Estrogen Negative Breast Cancer, Bethesda, MD

2003 Invited Speaker, Keystone Symposium, Molecular Targets in Cancer Therapy, Banff, Alberta, Canada

2004 Platform Presentation, Twentieth Oncogene Meeting, Frederick, MD

2006 Invited Lecture, Timberline Symposium, Timberline, OR

2008 Faculty Speaker, HHMI Medical Fellows Meeting, Chevy Chase, MD

2009 Speaker, Stand Up To Cancer (SU2C) Breast Cancer Dream Team Meeting, AACR, Philadelphia, PA

2010 Invited Speaker, Keystone Symposium on Cell Death. Vancouver, BC, Canada

2010 Co-chair and Invited Speaker, National Cancer Institute Workshop on Autophagy and Cancer, Bethesda, MD

2010 Keynote Speaker, 8th Annual Clinical Investigator Trainee (CIST) Meeting, HHMI, Chevy Chase, MD

2011 Invited Speaker, NCI/CCSB Workshop on Systems

Biology of Tumor Dormancy, Boston, MA

2013 Invited Plenary Speaker, Applied Pharmaceutical Toxicology Meeting, Genentech Inc., South San Francisco, CA

2014 Invited Speaker, National Breast Cancer Coalition Artemis Meeting on Tumor Dormancy, Calistoga, CA

2014 Invited Speaker, National Breast Cancer Coalition Leadership Summit, Alexandria, VA

2014 Speaker, Samuel Waxman Cancer Research Foundation Annual Meeting, New York, NY

2014 Invited Speaker, Keystone Symposium on Autophagy and Disease, Austin, TX

2015 Invited Speaker and Co-organizer, Keystone Symposium on Autophagy, Breckenridge, CO

2015 Invited Plenary Speaker, PISA 2015, Recent Advances in Cell Injury, Inflammation, and Neoplasia, American Society for Investigative Pathology, Baltimore, MD

REGIONAL AND OTHER INVITED PRESENTATIONS

2003 Invited Lecture, University of Vermont Cancer Center Symposium, Burlington, VT

2003 Invited Lecture, Society for Developmental Biology, Woods Hole, MA

2004 Invited Lecture, Department of Molecular Biomedical Research, University of Ghent, Belgium

2004 Invited Lecture, Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, Newark, NJ

2004 Invited Lecture, Cancer Biology and Genetics Program, Sloan-Kettering Institute, New York, NY

2005 Seminar, UCSF Rock Hall Research In Progress Series

2005 Speaker, UCSF BMS Graduate Program Retreat, Granlibaken, CA

2006 Seminar, UCSF Breast Oncology Program, San Francisco, CA

2006 Seminar, UCSF Molecular Medicine Program Seminar Series, San Francisco, CA

2006 Invited Speaker, UCSF Cancer Center Seminar Series, San Francisco, CA

2007 Invited Lecture, Wayne State University Department of Pharmacology (Mar 2007), Detroit, MI

2007 Invited Lecture, Northwestern University, Department of Endocrinology and Molecular Medicine (Jan 2007), Chicago, IL

2007 Invited Lecture, Stanford University, Geriatric Research, Education, and Clinical Center, Palo Alto, CA

2008 Invited Lecture, Glaxo Smith Kline Pharmaceuticals, Collegeville, PA

2008 Invited Keynote Speaker, Symposium on Cell Death, University of Colorado Heath Sciences Center, Denver, CO

2008 Invited Keynote Speaker, University of California Davis Medical Center Breast Cancer Symposium, Sacramento, CA

2008 Invited Lecture, UNMC Eppley Comprehensive Cancer Center, Omaha, NE

2009 Invited Lecture, Han-Mo Koo Memorial Seminar, Van Andel Institute, Grand Rapids,MI

2009 Invited Lecture, San Francisco Veteran's Administration Medical Center, San Francisco, CA

2009 Invited Lecture, Buck Institute for Age Research, Novato, CA

2009 Invited Lecture, Diller Family Cancer Center Building Inaugural Scientific Symposium, San Francisco, CA

2009 Invited Lecture, Georgia Institute of Technology School of Biology, Atlanta, GA

2009 Invited Lecture, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA

2010 Invited Lecture, Lerner Research Institute and Taussig Cancer Center, Cleveland Clinic, Cleveland, OH

2010 Invited Lecture, Ontario Cancer Institute, University of Toronto, Toronto, ON, Canada

2010 Invited Lecture, Clontech Laboratories, Mountain View, CA

2010 Invited Lecture, University of Massachusetts Medical Center Department of Cancer Biology, Worchester, MA

2010 Invited Lecture, University of Colorado Denver Cancer Center, Denver, CO

2010 Invited Lecture, San Francisco State University Department of Biology Seminar Series, San Francisco, CA

2010 Invited Lecture, UCSF Heme/Onc Research Seminar Series, San Francisco, CA

2010 Invited Seminar, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD

2010 Speaker and Co-organizer, Helen Diller Family Comprehensive Cancer Center Workshop on Metabolism and Cancer, UCSF, San Francisco, CA

2011 Invited Speaker, Novartis Institute of Biomedical Research, Cambridge, MA

2011 Speaker, UCSF Biomedical Sciences Graduate Program Retreat, Granlibakken, CA

2012 Invited Speaker, University of Southern California Keck School of Medicine, Cellular Homeostasis Lecture Series, Los Angeles, CA

2012 Invited Seminar, Department of Oncological Sciences, Mount Sinai School of Medicine, New York, NY

2012 Invited Speaker, UCSF Cancer Center Seminar Series, San Francisco, CA

2012 Invited Speaker, University of Minnesota Department of Biochemistry, Molecular Biology, and Biophysics Seminar Series, Minneapolis, MN

2012 Invited Speaker, Molecular Pharmacology & Chemistry Research Seminar Series Memorial Sloan-Kettering Cancer Center, New York, NY

2012 Invited Seminar, Department of Biological Sciences and Center for the Study for Gene Structure and Function, Hunter College, City University of New York, New York, NY

2012 Invited Seminar, Cleave Biosciences, Burlingame, CA

2012 Invited Speaker, 1st Annual Helen Diller Family Cancer Center Annual Retreat, Santa Cruz, CA

2012 Invited Seminar, Department of Physiology, University of Texas Health Science Center, San Antonio, TX

2013 Invited Keynote Speaker, 2013 Vancouver Autophagy Symposium, Vancouver, BC, Canada

2013 Invited Seminar, Department of Cell and Developmental Biology, Oregon Health & Sciences University, Portland, OR.

2013 Invited Speaker, UCSF Breast Oncology Program, San Francisco, CA

2013 Invited Seminar, Amgen Department of Oncology, San Francisco, CA

2014 Invited Seminar, Institut Pasteur, Paris, France

2014 Invited Seminar, Program in Cancer Biology, University of

Hawaii Cancer Center, Honolulu, HI

2014 Invited Seminar, University of Chicago, Ben May Cancer Biology Program, Chicago, IL

2014 Invited Seminar, Distinguished Speaker Series, Department of Translational Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX

2015 Invited Speaker, University of Cincinnati, Department of Cancer Biology, Cincinnati, OH

CONTINUING EDUCATION COURSES ATTENDED

2003 Osler Pathology Review Course, Tampa, FL

2004 San Antonio Breast Cancer Symposium, San Antonio, TX

2011 AACR Annual Meeting, Orlando, FL

2013 ASCI Annual Meeting, Chicago, IL

GOVERNMENT AND OTHER PROFESSIONAL SERVICE

2008 -	Hong Kong Research Grant Council	External grant reviewer
2008 -	National Medical Research Council, Singapore	External grant reviewer
2008 -	Austrian Science Fund	External grant reviewer
2008 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2008 -	United Kingdom Breast Cancer Campaign	External grant reviewer
2009 -	National Medical Research Council, Singapore	External grant reviewer
2009 -	Hong Kong Research Grant Council	External grant reviewer
2009 -	Health Research Board of Ireland	External grant reviewer
2009 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2009 -	NIH Challenge Grants (RC1)	Ad hoc Reviewer (Stage 1)
2010 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2010 -	French National Research Agency	External grant reviewer
2010 -	New Jersey Cancer Commission	Program project reviewer
2010 -	Health Research Board of Ireland	External grant reviewer
2010 -	Medical Research Council, United Kingdom	External grant reviewer
2010 -	NIH Director's Challenge (RC4)	Ad hoc Reviewer (Stage 1)
2011 -	Cancer Research UK (CRUK)	External grant reviewer

2011 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2011 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2011 -	Wellcome Trust, United Kingdom	External grant reviewer
2011 -	Danish Cancer Society	External grant reviewer
2011 -	NIH MBPP (Membrane Biology and Protein Processing) Study Section	Ad hoc Reviewer
2011 -	Hong Kong Research Grant Council	External grant reviewer
2012 -	Cancer Research UK (CRUK)	External grant reviewer
2012 -	National Medical Research Council, Singapore	External grant reviewer
2012 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2012 -	Hong Kong Research Grant Council	External grant reviewer
2012 -	Howard Hughes Medical Institute (HHMI) Medical Student Fellows Program	Fellowship program reviewer
2012 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2012 -	Luxemborg National Research Fund (FNR)	External grant reviewer
2012 -	NIH TCB (Tumor Cell Biology) Study Section	Ad hoc reviewer
2012 -	Biotechnology and Biomedical Sciences Research Council (BBSRC), United Kingdom	External grant reviewer
2013 -	Swiss National Science Foundation, Switzerland	External grant reviewer
2013 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2013 -	National Medical Research Council, Singapore	External grant reviewer
2013 -	Hong Kong Research Grant Council	External grant reviewer
2013 -	NIH SBIR Grants Study Section	Ad hoc reviewer
2013 -	NIH TCB (Tumor Cell Biology) Study Section	Ad hoc reviewer
2013 -	NIH Cancer Biology Special Emphasis Panel	Ad hoc reviewer
2013 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2013 - 2019	NIH TCB (Tumor Cell Biology) Study Section	Permanent member
2014 -	Department of Defense Breast Cancer Research Program	Ad hoc programmatic reviewer, Integration Panel
2014 -	Health Research Board of Ireland	External grant reviewer
2014 -	Italian Association of Cancer Research (AIRC)	External grant reviewer
2014 -	Deutsche Krebshilfe (German Cancer Aid)	External grant reviewer

UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

UCSF CAMPUS-WIDE

2009 - present	UCSF Graduate Division Community Building Workshop (formerly called Diversity Workshop)	Small group leader
2011 - present	UCSF Diversity Committee	Member

SCHOOL OF MEDICINE

2005 - 2010	Interviewer, Molecular Medicine Fellowship Program	
2008 - 2009	Stewart Trust Grant Review Committee Member	HDFCCC
2009 -	Laboratory Medicine (Breast Oncology Program Director) Search Committee	Member (Recruited Laura Van't Veer)
2012 - present	ACS Individual Research Award Grant Review Committee Member	HDFCCC
2013 - present	Research Allocation Program Training (RAPtr) Committee	Member
-		
-	Service for Biomedical Sciences (BMS) Graduate Program:	
2005 - present	Interviewer, BMS Graduate Program	
2006 - present	Biomedical Sciences (BMS) Graduate Program Admissions Committee	Member
2007 - present	BMS Seminar Committee	Member
2007 - present	BMS Student Advisory Committee	Member
2008 - present	Under-represented Minority Subcommittee, BMS Admissions Committee	Member
2009 - present	BMS Graduate Program Internal Selection Committee for Fellowships and Awards	Member
2010 - present	Summer Research Training Program, Placement of accepted students into UCSF labs	
2012 - present	Guidelines Committee for Preliminary Qualifying Exam, Biomedical Sciences Program (with Allan Balmain and Tony Defranco)	Member
2014 - 2015	BMS Admissions Committee	Chairperson

DEPARTMENTAL SERVICE

2005 - 2007	Departments of Pathology and Lab Medicine Website	Member
2005 - present	Interviewer, Anatomic Pathology Residency	Interviewer

	Program	
2008 - 2011	Pathology/Diller Cancer Center Faculty Search Committee	Member (Recruited Bradley Stohr and Scott Seeley)
2009 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant (Postdoctoral Fellows) Selection	Ad-Hoc Reviewer
2011 - present	Pathology Bridge Funding Committee	Member
2012 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2012 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant (Postdoctoral Fellows) Selection	Ad-Hoc Reviewer
2013 -	Departments of Anatomy and Pathology Faculty Search Committee	Member (Recruited Eric Snyder)
2013 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2013 -	Dept of Anatomy (Werb) T32 Cancer Research Training Grant for Post-doctoral Fellows	Associate Director
2014 -	Pathology and Laboratory Medicine Physician Scientist Pathway Research Day	Faculty Speaker
2013 - present	Department of Pathology Faculty Search Committee	
-		
-	Georgia Tech & Harvard Medical School	
1998 - 2001	Regional Selection Committee, Georgia Tech President's Scholarship Program	
2003 - 2003	Organizing committee, Harvard Medical School on "Open Access" Publishing	

PUBLIC SERVICE

2007 -	Annual Biomedical Research Conference for Minority Students Poster session judge and BMS minority recruitment, Austin, TX
2007 -	ReachMD "Clinician's Roundtable" Radio Interview
2007 -	Georgia Institute of Technology President's Scholarship Program Keynote Speaker, PSP Annual Luncheon, Atlanta, GA
2009 -	Interviewed for Science Careers (from the journal Science) for article on "Redefining Tenure at Medical Schools" by Chesea Wald.
2009 -	Interviewed for "Living History" Video, Georgia Institute of Technology Alumni Association

2010 - Panelist, Career Panel Discussion 8th Annual Clinical Investigator Trainee Meeting, NIH, Bethesda, MD.

2013 - Dinner Speaker, Regional Meeting of HHMI Medical Student Research Fellows, San Francisco, CA.

SUMMARY OF SERVICE ACTIVITIES

My university service is primarily devoted to the Biomedical Sciences (BMS) Graduate Program and toward the recruitment and retention of underrepresented minority students into graduate programs at UCSF. Since 2012, I have served on the RAPtr Committee for the School of Medicine, which is responsible for the review and selection of proposals from medical students to receive funding to conduct summer and year-long research projects.

For 2014-15, I will serve as the Chair of the Admissions Committee for the BMS Graduate Program.

TEACHING AND MENTORING

TEACHING

FORMAL SCHEDULED CLASSES FOR UCSF STUDENTS

Qtr	Academic Yr	Course Number and Title	Teaching Contribution	Units	Class Size
Fall	2000 - 2004	Harvard Medical School Medical Student Pathology	Lab Instructor	0	8
FA	2005 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2006 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2007 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2008 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2010 -	BMS260, BMS Cell Biology	Discussion Leader	4	8
FA	2006 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2007 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2008 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2009 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2010 -	Infection, Immunity, and Inflammation	Lab Instructor	0	25
FA	2012 -	Infection, Immunity and Inflammation	Lab Instructor	0	25
FA	2013 -	Infection, Immunity and Inflammation	Lab Instructor		25
FA	2014 -	Infection, Immunity, and Inflammation	Lab Instructor		25

Qtr	Academic Yr	Course Number and Title	Teaching Contribution	Units	Class Size
FA	2006 -	Cancer	Discussion Leader	0	15
FA	2007 -	Cancer	Discussion Leader	0	15
FA	2008 -	Cancer	Discussion Leader	0	15
FA	2009 -	Cancer	Discussion Leader	0	15
FA	2010 -	Cancer	Discussion Leader	0	15
FA	2011 -	Cancer	Discussion Leader		15
FA	2013 -	Cancer	Discussion Leader		15
FA	2014 -	Cancer	Discussion Leader		15
WI	2007 -	Biochem 297, Molecular Pathology/Biology of Neoplasia	Lab Instructor	0	15
WI	2009 -	Biochem 297, Molecular Pathology/Biology of Neoplasia	Lab Instructor	0	15
WI	2007 -	Life Cycle/Epilogue	Lab Instructor	0	24
WI	2008 -	Life Cycle/Epilogue	Lab Instructor	0	24
WI	2009 -	BMS230, Molecular and Cellular Biology of Cancer	Lecturer (1-2 lectures)	4	25
FA	2010 -	BMS230, Molecular and Cellular Biology of Cancer	Lecturer (1-2 lectures)	4	25
FA	2011 -	Physio 181, Demystifying Medicine	Lecturer (1 lecture)	2	25
FA	2012 -	BMS230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (1 lecture and 1 discussion section)	4	8
FA	2013 -	BMS 230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (2 lectures and 1 discussion section)	4	25
WI	2014 -	Physio 181, Demystifying Medicine	Lecturer (1 lecture)	2	25
FA	2014 -	BMS 230, Advanced Topics In Cancer Research	Co-Course Director and Lecturer (2 lectures and 1 discussion section)	4	25

INFORMAL TEACHING

2005 - 2006 BMS Journal Club Faculty Coach (Guided paper discussions and oral presentations for 3 graduate students (3 hours per student)), University of California, San Francisco

2005 - present Autopsy service attending pathologist (Supervised 2-4 residents/medical students six weeks per year), UCSF Medical Center

2006 - 2007 Qualifying exam committee member (Served on qualifying exam committee for 4 BMS graduate students (7-10 hrs per student)), University of California, San Francisco

2006 - 2007 BMS Journal Club Faculty Coach (Guided oral presentations for 4 graduate students (2-3 hours per student)), University of California, San Francisco

2006 - 2006 Discussion Leader, "Mentoring Mania" Symposium, UCSF (Led one time (1 hour) discussion with 5 post-docs), UCSF Medical Center

2007 - 2008 Qualifying exam committee member (Served on qualifying exam committee for 1 BMS and 1 CCB graduate student (7-10 hrs per student)), University of California, San Francisco

2007 - 2008 Chair, qualifying exam committee (Chair of qualifying exam committee for Seth Bechis (BMS/MSTP student), 10 hrs total), University of California, San Francisco

2007 - 2008 BMS Journal Club Faculty Coach (Guided oral presentations for one graduate student (2-3 hours per student)), University of California, San Francisco

2007 - 2009 Thesis Advisory Committee, Cathy Collins (Ph.D. candidate, MSTP/BMS) (Meet 1-2 times yearly, 2 hrs per meeting), University of California, San Francisco

2007 - 2010 Thesis Advisory Committee, (Kate Nestor, Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco

2007 - 2011 Thesis Advisory Committee, Daniel Garcia (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2007 - 2011 Thesis Advisory Committee, Brandon Tavshajian (Ph.D. candidate, CCB) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2008 - 2009 Chair, Qualifying exam committee (Chair of qualifying exam committee for Sarah Gierke (BMS), 10h total), University of California San Francisco

2008 - 2009 Faculty Mentor for NSF and BMS260 proposals, BMS Graduate Program (Advised two first-year BMS sutdents in writing NSF and BMS260 proposals (10 hours per student)), University of California San Francisco

2008 - 2009 BMS Journal Club Faculty Coach (Guided oral presentations for five graduate students (2-3 hours per student)), University of California, San Francisco

2008 - 2009 Faculty Mentor for BMS225A proposal, BMS Graduate Program (Advised one first-year BMS sutdents in writing BMS225A proposal (10 hours total)), University of California San Francisco

2008 - 2009 Chair, Qualifying exam committee (Chair of qualifying exam committee for Jonathan Chou (MSTP/BMS), 10 hrs total), University of California, San Francisco

2009 - 2010 Qualifying exam committee member (Served on qualifying exam committee for 2 BMS graduate students (7-10 hrs per student)), University of California, San Francisco

2009 - 2010 BMS Journal Club Faculty Coach (Guided oral presentations for three graduate students (2-3 hours per student)), University of California, San Francisco

2009 - 2012 Thesis Advisory Committee, Jonathan Chou (Ph.D. candidate, MSTP/BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2010 - 2013 Chair, Thesis Advisory Committee, Lionel Lim (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), University of California, San Francisco School of Medicine

2009 - 2010 Chair, Qualifying exam committee (Chair of qualifying exam committee for Megan Salt (BMS), 10 hrs total), University of California, San Francisco

2010 - 2011 Qualifying Exam committee member (Serve on qualifying exam committee for 1 BMS, 1 PIBS, 1 Biophysics, and 1 Neuroscience graduate student, 7-10 hrs per student), University of California, San Francisco.

2010 - 2011 BMS Journal Club Faculty Coach (Guided oral presentations for two graduate students (2-3 hours per student)), University of California, San Francisco

2011 - 2012 Qualifying Exam committee member (Serve on qualifying exam committee for 1 DSCB Student and 2 BMS Students, 7-10 hrs per student), University of California, San Francisco.

2011 - 2012 BMS and CCB Journal Club Faculty Coach (Guided oral presentations for three graduate students (2-3 hours per student)), University of California, San Francisco

2012 - present Thesis Advisory Committee, Si-Han Chen (Ph.D. candidate, Biophysics) (1 meeting/year, 2h per mtg), UCSF

2012 - present Thesis Advisory Committee, Julia Marguiles (Ph.D. candidate, Neuroscience) (1 meeting/year, 2h per mtg), UCSF

2012 - present Thesis Advisory Committee, Mike Ando (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2012 - present Thesis Advisory Committee, Brittany Anderton (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

2012 - 2013 BMS Journal Club Faculty Coach (Guided oral presentations for one graduate students (2-3 hours per student)), University of California, San Francisco

2012 - 2013 Qualifying Exam committee member (Serve on qualifying exam committee for 1 UCB/UCSF Bioengineering Student), University of California, San Francisco.

2012 - 2013 Faculty Mentor for BMS225A proposal, BMS Graduate Program (Advised one first-year BMS student in writing BMS225A proposal (10 hours total)), University of California San Francisco

2012 - 2013 Chair, Qualifying exam committee (Chair of qualifying exam committee for Gianne Souza (BMS), 10h total), University of California San Francisco

2013 - present Chair, Thesis Advisory Committee, Florie Charles (Ph.D. candidate, BMS) (1 meeting /year, 2h per mtg), UCSF

2013 - present Thesis Advisory Committee, Darien Reed (Ph.D. candidate, MSTP/Tetrad) (1 meeting/year, 2h per mtg), UCSF

2013 - present Thesis Advisory Committee, Alec Cerchiari (Ph.D. candidate, Bioengineering Student) (1 meeting/year, 2h per mtg), UCB/UCSF Bioengineering Program.

2013 - present Chair, Thesis Advisory Committee, Renee Rivas (Ph.D. candidate, MSTP/BMS) (1 meeting/year, 2h per mtg), University of California San Francisco

2013 - Invited Speaker, UCSF Medical Scientist Training Program (MSTP) Grand Rounds, University of California, San Francisco

2012 - 2013 Chair, Qualifying exam committee (Chair of qualifying exam committee for Christine Sheridan (BMS), 10h total), University of California San Francisco

2013 - 2014 Chair, Qualifying exam committee (Chair of qualifying exam committee for Amanda Paulson (BMS), 10h total), University of California San Francsico

2013 - 2014 BMS Journal Club Coach (Guided oral presentations for three BMS graduate students, 2-3 hours per student), University of California San Francisco

2013 - 2014 Department of Pathology MOD Conference Mentor (Guided oral presentation by Manana Kvezerelli, Anatomic Pathology Resident, 1-2 hrs), University of California San Francisco

2013 - 2014 Qualifying Exam committee member (Served on qualifying exam committee for 1 BMS, 1 Tetrad Student and 1 Bioengineering), University of California, San Francisco.

2013 - 2014 Chair, Qualifying exam committee (Chair of qualifying exam committee for David Pardo (BMS), 10 h total), University of California, San Francisco.

2014 - 2015 BMS Journal Club Coach (Guided oral presentations for one BMS graduate student, 2-3 hours per student), University of California San Francisco

2014 - 2015 Thesis Advisory Committee, Joe Udoechu (Ph.D. candidate, BMS) (1 meeting/year, 2h per mtg), UCSF

TEACHING NARRATIVE

My formal teaching expertise encompasses the areas of cell biology, cancer biology, and pathology.

Graduate school: I primarily teach graduate level courses for the Biomedical Sciences Graduate Program. I have served as a discussion leader to a group of 8-10 first year students in the BMS 260 Cell Biology course and given lectures and led discussions for the BMS Cancer Biology (BMS 230) course.

I also advise graduate students in the BMS program in my laboratory, both rotation students and students working toward a thesis in my laboratory. Currently, three BMS graduate students are pursuing a thesis in my laboratory. I have served as a chair or as a member of multiple Qualifying Exam Committees for 2nd year UCSF graduate students (in the BMS, PIBS, CCB, Neuroscience, Bioengineering and Biophysics programs) and currently serve as a chair or member of eight Thesis Advisory Committees for advanced graduate students in the BMS, Neuroscience and Biophysics Programs. In

addition, I regularly serve as a faculty coach to students for their oral presentations at the BMS Journal Club, and as a mentor for student's written proposals in various BMS courses.

Course DIrector for BMS230, Advanced Topics In Cancer Research: In 2012, I became the co-course director of the BMS 230 Cancer Biology course with Professor Martin McMahon at HDFCCC; we reorganized the curriculum, previously a survey course, into a series of focused, in-depth advanced topics in cancer research. These topics will change on a yearly basis in order to cover timely issues in cancer biology. We are leading this course as an elective every fall to first and second year graduate students at UCSF.

Medical School: In the medical school curriculum at UCSF, I teach as a lab instructor in the Immunology Block (I3) and small group discussion leader for the pathology section in the Cancer Block (M3). I also supervise and teach anatomic pathology residents and medical students as an attending in the autopsy service at Moffitt Hospital at UCSF (6 weeks per year).

MENTORING

PREDOCTORAL STUDENTS SUPERVISED OR MENTORED

Dates	Name	Program or School	Role	Current Position
2001 - 2005	Carolyn Wrobel	Harvard Medical School	Advisor for rotation and during graduate thesis work, Brugge Lab	Assistant Professor, Depaul University
2001 - 2001	Diedra Wrighting	Harvard Medical School	Rotation Advisor, Brugge Lab	Research Scientist, Broad Institute, MIT
2001 - 2001	Sean Beausoliel	Harvard Medical School	Rotation Advisor, Brugge Lab	Research Scientist, Cell Signaling Technologies
2005 - 2007	Chris Fung	University of California, San Francisco	Supervised post undergraduate research	Medical Student, University of Pittsburgh
2006 - 2011	Lilly Radoshevich	University of California, San Francisco	Thesis Advisor, BMS Graduate Student	EMBO Post-doctoral Fellowship (Pascal Cossart, HHMI and Pasteur Institut, Paris, France)
2006 - 2011	Rebecca Lock	University of California, San Francisco	Rotation Advisor/Thesis Advisor	CTF Young Investigator Post-doctoral Fellowship (Karen Cichowski, Harvard Medical School)
2007 - 2013	Eduardo Salas	University of California, San Francisco	Supervised post undergraduate research	Research Scientist, Gilead Corporation, Foster City, CA
2007 - 2009	Cynthia Jimenez	University of California, San Francisco School of	M.D. With Thesis Committee Member	Resident, UCSF Pathology

Dates	Name	Program or School	Role	Current Position
		Medicine		
2008 - 2008	Ada Li	University of California, San Francisco	Summer Research Mentor (SEP High School Program)	Undergraduate, UCLA
2008 - present	Candia Kenific	University of California, San Francisco	Rotation advisor/Thesis Advisor	BMS Graduate Student
2009 - present	Lyndsay Murrow	University of California, San Francisco	Rotation advisor/Thesis Advisor	BMS Graduate Student
2009 - 2013	Laura Westrake	Van Andel Research Institute	Thesis committee member (external advisor)	Post-doc, Gia Voltz Lab, University of Colorado Boulder
2009 - 2009	Estefania Fernandez	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program)	MD/PhD (MSTP) Program, Washington Univ-St. Louis
2010 - 2010	Nuria Eritja	University of California, San Francisco	Short term stay advisor	Post-doc, Spain
2011 - 2011	Shivali Gupta	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program)	MPH, University of California, Berkeley
2011 - 2012	Sanaaz Sadegh	University of California, San Francisco	Advisor, International Undergraduate Internship	Graduate Student, University of Southern California
2012 - present	Hanna Kuznetsov	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student
2012 - 2013	Kimberley Woo	University of California, Berkeley	Undergraduate Research Advisor	Undergraduate, UC Berkeley
2012 - 2013	Jordan Wu	University of California, Berkeley	Undergraduate Research Advisor	Junior Specialist, Debnath Lab, UCSF
2013 - present	Jordan Wu	University of California, San Francisco	Supervised post-undergraduate research	Junior Specialist, Debnath Lab, UCSF
2013 - present	Juliet Goldsmith	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student
2013 -	Jeff Chukwuneke	University of California, San Francisco	Summer Research Mentor (SRTP Undergraduate Program-Amgen Scholars)	Medical Student, Columbia University College of Physicians and Surgeons
2014 -	Caroline Park	Albert Einstein College of Medicine, Bronx, NY	External examiner, PHD thesis	MSTP Student, Cuervo Lab, Albert Einstein
2014 - present	Timothy Marsh	University of California, San Francisco	Rotation advisor/Thesis advisor	BMS Graduate Student

Dates	Name	Program or School	Role	Current Position
2014 -	Rocio Saavedra	University of Puerto Rico, PR	Summer Research Mentor (SRTP Undergraduate Program-NSF)	Undergraduate, University of Puerto Rico
2014 - present	Florie Charles	University of California, San Francisco	Thesis Advisor (Co-mentor with Dr. Brad Stohr)	BMS Graduate Student

POSTDOCTORAL FELLOWS AND RESIDENTS DIRECTLY SUPERVISED OR MENTORED

Dates	Name	Fellow	Faculty Role	Current Position
2007 - 2011	Nan Chen	University of California, San Francisco	Postdoctoral Advisor	Research scientist, Minnesota.
2007 - 2010	Kimberley Evason	University of California, San Francisco School of Medicine	Advisor, UCSF Molecular Medicine Program	Post-doc, UCSF Dept of Tissue Biology (Andre Goga)
2007 - 2010	W. Patrick Devine	University of California, San Francisco School of Medicine	Advisor, UCSF Molecular Medicine Program	Post-doc, Gladstone Institute (Benoit Bruneau)
2007 - present	Ritu Malhotra	University of California, San Francisco	Postdoctoral Advisor	Postdoctoral fellow
2008 - present	Srirupa Roy	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2011 - present	Jasvinder Kaur	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2011 - present	Jennifer Rudnick	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2013 - present	Andrew Leidel	University of California, San Francisco School of Medicine	Postdoctoral Advisor	Post-doctoral fellow
2013 - present	David Solomon	University of California, San Francisco School of Medicine	Sponsor, UCSF Physician Scientist Scholar Program Application	Resident, Anatomic Pathology (PGY2)
2014 - present	Craig Forrester	University of California, San Francisco School of Medicine	Member, Scholarship Oversight Committee, UCSF Pediatrics	Pediatric Heme/Onc Fellow and Post-doc, Ruggero Lab

FACULTY MENTORING

Dates	Name	Position While Mentored	Mentoring Role	Current Position
2012 -	Andrew Hseih	Adjunct Instructor	Career development and K08 application mentoring	Adjunct Instructor, UCSF Dept of Medicine
2014 - present	Eric Snyder	Assistant Professor	Department of Pathology Faculty Mentor	Assistant Professor, UCSF Dept of Pathology
2014 - present	Anatoly Urisman	Clinical Instructor	Department of Pathology Faculty Mentor	Clinical Instructor, UCSF Pathology

MENTORING NARRATIVE

My own laboratory is dedicated to the education of post-doctoral fellows, graduate students, and undergraduate students. My principal mentoring role is as a PhD advisor to graduate students in the BMS program. Currently, four BMS graduate students are pursuing a thesis in my laboratory. In addition, I currently have five post-doctoral fellows in my lab, and regularly host rotation students, summer undergraduate students, and visiting graduate students and fellows for short-term stays in my lab. My record of mentorship, especially with regard to graduate students, is best evidenced by the track record of my trainees in obtaining first-author publications as well as competitive fellowships and awards. Notably, both of my first two PhD graduates, Rebecca Lock and Lilly Radoshevich, are pursuing academic post-doctoral fellowships at Harvard Medical School and Institut Pasteur, Paris respectively.

In the Department of Pathology, I serve as a career advisor to residents and fellows interested in becoming academic experimental pathologists. In 2013, I began service as Associate Director (Basic Science) for the "Molecular and Cellular Mechanisms of Cancer" T32 Training Grant (T32 CA108462, PI: Zena Werb), a multi-departmental training grant for post-doctoral fellows pursuing cancer research at UCSF and the HDFCCC.

OTHER

COMPETITIVE FELLOWSHIPS AND AWARDS OBTAINED BY TRAINEES:

Lilly Radoshevich (PhD Student), Sandler/Genentech Predoctoral Fellowship, 2007-08.

Rebecca Lock (PhD Student), DOD Breast Cancer Predoctoral Fellowship, 2008-11.

Rebecca Lock (PhD Student), CRCC Predoctoral Fellowship, 2008-09 (declined).

Lilly Radoshevich (PhD Student), Phi Beta Kappa Scholarship, 2010.

Lilly Radoshevich (PhD Student), Keystone Scholarship (Travel award), 2010.

Lyndsay Murrow (PhD Student), NSF Graduate Fellowship, 2010-2013.

Candia Kenific (PhD Student), Genentech Predoctoral Fellowship, 2010-12.

Rebecca Lock (PhD Student), HDFCCC Student Invitee to Cancer Molecular Therapeutics Research Association Conference, 2011.

Candia Kenific (PhD Student), University of California Cancer Research Coordinating Committee Fellowship, 2011-12.

Rebecca Lock (PhD Student), DOD Era of Hope Meeting Outstanding Poster Award, 2011

Jennifer Rudnick (Post-doc), NIH T32 Training Grant Recipient, 2011-13.

Candia Kenific (PhD Student), NRSA Graduate Student Fellowship (F31 CA167905), National Cancer Institute, 2012-2015

Jennifer Rudnick (Post-doc), ACS Postdoctoral Fellowship Recipient, 2013-16 (terminated early to start DOD BCRP Fellowship).

Jennifer Rudnick (Post-doc), DOD Breast Cancer Postdoctoral Fellowship Recipient, 2013-16.

Hanna Kuznetsov (PhD Student), NSF Graduate Fellowship, 2013-2016.

Jasvinder Kaur (Post-doc), Keystone Scholarship (Travel award), 2014.

Hanna Kuznetsov (PhD Student), Discovery Fellow, UCSF Graduate Division, 2014.

Juliet Goldsmith (PhD Student), NSF Graduate Fellowship, 2014-2017.

Hanna Kuznetsov (PhD Student), HHMI-CTSI GEMS Fellowship, 2014 (declined)

Juliet Goldsmith (PhD Student), Discovery Fellow, UCSF Graduate Division, 2014.

Florie Charles (PhD Student), Discovery Fellow, UCSF Graduate Division, 2014.

SUMMARY OF TEACHING AND MENTORING HOURS

2013 - 2014	290 total hours of teaching (including preparation)
	Formal class or course teaching hours: 40 hours
	Informal class or course teaching hours: 250 hours
	Mentoring hours: 150 hours
	Other Hours:
2014 - 2015	290 total hours of teaching (including preparation)
	Formal class or course teaching hours: 40 hours
	Informal class or course teaching hours: 250 hours
	Mentoring hours: 150 hours

Other Hours:

2015 - 2016 Total anticipated hours of teaching: 400 hours

RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS

CURRENT

W81XWH-11-1-0130 (Principal Investigator) 09/2011 - 08/2016
DOD BCRP Era of Hope Scholar Award
Eliminating Late Recurrence to Eradicate Breast Cancer.

RO1 CA126792 (Principal Investigator) 02/2009 - 01/2015
NIH
Autophagy and Epithelial Cell Fate During Anoikis and 3D Morphogenesis.

R01 CA188404 (Principal Investigator, (MPI: Debnath and Bergers)) 09/2014 - 06/2019
NIH/NCI
Autophagy as a microenvironmental regulator of tumorigenesis and resistance.

SWCRF (Prinicipal Investigator) 07/2013 - 06/2015
Samuel Waxman Cancer Research Foundation--RFA on Reprogramming the Cancer Cell
Effects of Autophagy-Dependent Secretion on Carcinoma Differentiation

8-Ball (Principal Investigator) 9/2013 - 8/2016
8-Ball Foundation
Metabolic Adaptation In Gastrointestinal Stromal Tumor (GIST)

PAST

1KO8CA098419-01 (Principal Investigator) 2003 - 09
N.I.H.
Oncogenes and Luminal Apoptosis Within Mammary Acini

Culpeper Medical Scholar (Principal Investigator) 2006 - 09Debnath (PI)
Foundation
The Role and Regulation of Autophagy in Epithelial Cell Death

AACR \Genentech BioOncology Career Award (Principal Investigator) 2006 - 09Debnath (PI)

Foundation

The Role and Regulation of Autophagy Downstream of HER Family Pathways

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Foundation - Stewart Trust (Principal Investigator) 2007 - 08Debnath (PI)

Discovering New Autophagy Modulators For Cancer Chemotherapy

UC Cancer Research Coordinating Committee (Principal Investigator) 2007 - 08Debnath (PI)

CRCC

Autophagy During HMEC Agonescence

PBBR (Sandler) Integrative Research Award (Principal Investigator, Debnath and Ronen) 2009 - 10

Foundation

Defining the Metabolic Consequences of Autophagy Using Magnetic Resonance Spectroscopy

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TRDRP 18XT-0106 (Principal Investigator) 2009 - 2011

UCOP

Autophagy and K-Ras Mutant Lung Cancer Cells

RO1 CA126792-S1 (Principal Investigator) 2009 - 2011

NIH (ARRA Supplement)

Autophagy and Epithelial Cell Fate During Anoikis and 3D Morphogenesis. Recovery Act Supplement

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HHMI Early Career Award (Principal Investigator) 2006 - 07/30/2012

Regulation of ATG12 During Autophagy.

AACR/SU2C - Stand Up To Cancer Breast Cancer Dream Team (Co-Investigator) 2009 - 12

An Integrated Approach to Targeting Breast Cancer Molecular Subtypes and Their Resistance Phenotypes. $Salary support only (5% effort) direct/yr1

PBBR/Sanofi (Principal Investigator (Debnath and Xu)) 2011 - 2013

PBBR New Frontiers Research Award (Sanofi UCSF LIFTT Award)

Autophagy in Hypothalamus-Mediate Energy Balance and Obesity

UCSF Breast Oncology Program (Principal Investigator) 2013 - 2014

UCSF Breast Oncology Program/Breast Cancer SPORE Developmental Research Project

Autophagy-Dependent Secretion and Breast Cancer Progression

W81XWH-12-1-0505 (Principal Investigator) 2012 - 2014

DOD BCRP Innovator and Scholar Concept Award

Targeting Autophagy in the Tumor Stroma To Eradicate Breast Cancer

PEER REVIEWED PUBLICATIONS

1. Debnath J, Husain PA, May SW. Activation of an adrenergic pro-drug through sequential stereoselective action of tandem target enzymes. Biochem Biophys Res Commun. 1992 Nov 30; 189(1):33-9. PMID: 1449487

2. Husain PA, Debnath J, May SW. HPLC-based method for determination of absolute configuration of alpha-chiral amines. Anal Chem. 1993 May 15; 65(10):1456-61. PMID: 8517551

3. Van Etten RA, Debnath J, Zhou H, Casasnovas JM. Introduction of a loss-of-function point mutation from the SH3 region of the Caenorhabditis elegans sem-5 gene activates the transforming ability of c-abl in vivo and abolishes binding of proline-rich ligands in vitro. Oncogene. 1995 May 18; 10(10):1977-88. PMID: 7539119

4. Debnath J, Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, Schwartzberg PL. rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol. 1999 Feb; 19(2):1498-507. PMID: 9891083. PMCID: PMC116078

5. Schaeffer EM*, Debnath J*, Yap G, McVicar D, Liao XC, Littman DR, Sher A, Varmus HE, Lenardo MJ, Schwartzberg PL. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science. 1999 Apr 23; 284(5414):638-41. PMID: 10213685 ***co-first author.**

6. Schaeffer EM, Broussard C, Debnath J, Anderson S, McVicar DW, Schwartzberg PL. Tec family kinases modulate thresholds for thymocyte development and selection. J Exp Med. 2000 Oct 2; 192(7):987-1000. PMID: 11015440. PMCID: PMC2193319

7. Chamorro M, Czar MJ, Debnath J, Cheng G, Lenardo MJ, Varmus HE, Schwartzberg PL. Requirements for activation and RAFT localization of the T-lymphocyte kinase Rlk/Txk. BMC Immunol. 2001; 2:3. PMID: 11353545. PMCID: PMC31577

8. Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell. 2002 Oct 4; 111(1):29-40. PMID: 12372298

9. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods. 2003 Jul; 30(3):256-68. PMID: 12798140

10. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol. 2003 Aug; 5(8):733-40. PMID: 12844146

11. Debnath J, Walker SJ, Brugge JS. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol. 2003 Oct 27; 163(2):315-26. PMID: 14568991. PMCID: PMC2173511

12. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. Proc Natl Acad Sci U S A. 2004 Mar 9; 101(10):3438-43. PMID: 14993595. PMCID: PMC373480

13. Wrobel CN, Debnath J, Lin E, Beausoleil S, Roussel MF, Brugge JS. Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture. J Cell Biol. 2004 Apr 26; 165(2):263-73. PMID: 15117969. PMCID: PMC2172030

14. Chen GC, Lee JY, Tang HW, Debnath J, Thomas SM, Settleman J. Genetic interactions between Drosophila melanogaster Atg1 and paxillin reveal a role for paxillin in autophagosome formation. Autophagy. 2008 Jan; 4(1):37-45. PMID: 17952025

15. Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. Mol Biol Cell. 2008 Mar; 19(3):797-806. PMID: 18094039. PMCID: PMC2262959

16. Kim SH, Debnath J, Mostov K, Park S, Hunt CA. A computational approach to resolve cell level contributions to early glandular epithelial cancer progression. BMC Syst Biol. 2009; 3:122. PMID: 20043854. PMCID: PMC2814811

17. Kim SH, Park S, Mostov K, Debnath J, Hunt CA. Computational investigation of epithelial cell dynamic phenotype in vitro. Theor Biol Med Model. 2009; 6:8. PMID: 19476639. PMCID: PMC2696420

18. N'Diaye EN, Kajihara KK, Hsieh I, Morisaki H, Debnath J, Brown EJ. PLIC proteins or ubiquilins regulate autophagy-dependent cell survival during nutrient starvation. EMBO Rep. 2009 Feb; 10(2):173-9. PMID: 19148225. PMCID: PMC2637314

19. N'Diaye EN, Debnath J, Brown EJ. Ubiquilins accelerate autophagosome maturation and promote cell survival during nutrient starvation. Autophagy. 2009 May; 5(4):573-5. PMID: 19398896

20. Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, Galvan V. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces

amyloid-beta levels in a mouse model of Alzheimer's disease. PLoS One. 2010; 5(4):e9979. PMID: 20376313. PMCID: PMC2848616

21. Gupta A, Roy S, Lazar AJ, Wang WL, McAuliffe JC, Reynoso D, McMahon J, Taguchi T, Floris G, Debiec-Rychter M, Schoffski P, Trent JA, Debnath J*, Rubin BP*. Autophagy inhibition and antimalarials promote cell death in gastrointestinal stromal tumor (GIST). Proc Natl Acad Sci U S A. 2010 Aug 10; 107(32):14333-8. PMID: 20660757. PMCID: PMC2922542 ***co-senior author.**

22. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, Debnath J. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell. 2010 Aug 20; 142(4):590-600. PMID: 20723759. PMCID: PMC2925044

23. Fan QW, Cheng C, Hackett C, Feldman M, Houseman BT, Nicolaides T, Haas-Kogan D, James CD, Oakes SA, Debnath J, Shokat KM, Weiss WA. Akt and autophagy cooperate to promote survival of drug-resistant glioma. Sci Signal. 2010; 3(147):ra81. PMID: 21062993. PMCID: PMC3001107

24. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell. 2011 Jan 15; 22(2):165-78. PMID: 21119005. PMCID: PMC3020913

25. Mirzoeva OK, Hann B, Hom YK, Debnath J, Aftab D, Shokat K, Korn WM. Autophagy suppression promotes apoptotic cell death in response to inhibition of the PI3K-mTOR pathway in pancreatic adenocarcinoma. J Mol Med (Berl). 2011 Sep; 89(9):877-89. PMID: 21678117

26. Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J, Aguirre-Ghiso JA. PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. Mol Cell Biol. 2011 Sep; 31(17):3616-29. PMID: 21709020. PMCID: PMC3165554

27. Lee HS, Daniels BH, Salas E, Bollen AW, Debnath J, Margeta M. Clinical utility of LC3 and p62 immunohistochemistry in diagnosis of drug-induced autophagic vacuolar myopathies: a case-control study. PLoS One. 2012; 7(4):e36221. PMID: 22558391. PMCID: PMC3338695

28. Nedvetsky PI, Kwon SH, Debnath J, Mostov KE. Cyclic AMP regulates formation of mammary epithelial acini in vitro. Mol Biol Cell. 2012 Aug; 23(15):2973-81. PMID: 22675028. PMCID: PMC3408423

29. Chen N, Eritja N, Lock R, Debnath J. Autophagy restricts proliferation driven by oncogenic phosphatidylinositol 3-kinase in three-dimensional culture. Oncogene. 2013 May 16; 32(20):2543-54. PMID: 22777351. PMCID: PMC3470740

30. Avivar-Valderas A, Bobrovnikova-Marjon E, Alan Diehl J, Bardeesy N, Debnath J, Aguirre-Ghiso JA. Regulation of autophagy during ECM detachment is linked to a selective inhibition of mTORC1 by PERK. Oncogene. 2013 Oct 10; 32(41):4932-40. PMID: 23160380. PMCID: PMC3600386

31. Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J, Passegué E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. Nature. 2013 Feb 21; 494(7437):323-7. PMID: 23389440. PMCID: PMC3579002

32. Chen N, Debnath J. IkB kinase complex (IKK) triggers detachment-induced autophagy in mammary epithelial cells independently of the PI3K-AKT-MTORC1 pathway. Autophagy. 2013 Aug; 9(8):1214-27. PMID: 23778976. PMCID: PMC3748193

33. Damiano L, Stewart KM, Cohet N, Mouw JK, Lakins JN, Debnath J, Reisman D, Nickerson JA, Imbalzano AN, Weaver VM. Oncogenic targeting of BRM drives malignancy through C/EBPß-dependent induction of a5 integrin. Oncogene. 2014 May 8; 33(19):2441-53. PMID: 23770848. PMCID: PMC3960370

34. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-Dependent Production of Secreted Factors Facilitates Oncogenic RAS-Driven Invasion. Cancer Discov. 2014 Apr; 4(4):466-79. PMID: 24513958. PMCID: PMC3980002

35. Malhotra, R, Warne, J, Salas, E, Xu, AW, and Debnath, J. Loss of ATG12, but not ATG5, in pro-opiomelanocortin neurons exacerbates diet-induced obesity. Autophagy, Accepted for publication.

Review Articles

1. Czar MJ, Debnath J, Schaeffer EM, Lewis CM, Schwartzberg PL Biochemical and genetic analyses of the Tec kinases Itk and Rlk/Txk. Biochem Soc Trans. 29: 863-7, 11/2001.

2. Debnath J, Baehrecke EH, Kroemer G Does autophagy contribute to cell death? Autophagy. 1: 66-74 (Invited Review), Jul/2005.

3. Debnath J, Brugge JS Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer. 5: 675-88 (Invited Review), 9/2005.

4. Hebner C, Weaver VM, Debnath J Modeling Morphogenesis and Oncogenesis in Three-Dimensional Breast Epithelial Cultures. Annu Rev Pathol. (Invited Review), Sep/20/2007.

5. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clavé C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy. 4: 1 (Multi-author consortium) Nov/21/2007.

6. Debnath J. Detachment-induced autophagy during anoikis and lumen formation in epithelial acini. Autophagy. 4: (Invited commentary), Jan/7/2008.

7. Lock R, Debnath J Extracellular matrix regulation of autophagy. Curr Opin Cell Biol. 20: 583-8 (Invited Review), Oct/2008.

8. Debnath J Detachment-induced autophagy in three-dimensional epithelial cell cultures. Methods Enzymol. 452: 423-39 (Invited Review), 2009.

9. Kenific CM, Thorburn A, Debnath J Autophagy and metastasis: another double-edged sword. Curr Opin Cell Biol. 22(2): 241-5. (Invited Review), Nov/27/2009.

10. Chen N, Debnath J Autophagy and tumorigenesis. FEBS Lett. 584:1427-35 (Invited Review),Dec/24/2009.

11. Roy, S., Debnath J Autophagy and tumorigenesis. Sem. Immunopath. (Invited Review), June/30/2010.

12. Debnath J p66Shc and Ras: Controlling anoikis from the inside-out. Oncogene (Invited Commentary) 29(41): 5556-8. Oct/14/2010.

13. Rubin BP, Debnath J. Therapeutic implications of autophagy-mediated cell survival in gastrointestinal stromal tumor after treatment with imatinib mesylate. Autophagy. (Invited commentary) 6(8): 1190-1, Nov/15/2010.

14. Radoshevich L, Debnath J. ATG12-ATG3 and Mitochondria. Autophagy (Invited commentary). 7(1): 109-11, 2011, Epub Nov/24/2011.

15. Lock R, Debnath J. Ras, Autophagy and Glycolysis. Cell Cycle (Invited commentary) 10(10): 1516-7, May/15/2011.

16. Debnath J. The multifaceted roles of autophagy in tumors-implications for breast cancer. J Mammary Gland Biol Neoplasia. (Invited review) 2011 Sep; 16(3):173-87.

17. Klionsky DJ, Baehrecke EH, Brumell JH, Chu CT, Codogno P, Cuervo AM, Debnath J, Deretic V, Elazar Z, Eskelinen EL, Finkbeiner S, Fueyo-Margareto J, Gewirtz D, Jäättelä M, Kroemer G, Levine B, Melia TJ, Mizushima N, Rubinsztein DC, Simonsen A, Thorburn A, Thumm M, Tooze SA. A comprehensive glossary of autophagy-related molecules and processes (2nd edition). Autophagy (Multi-author consortium).2011 Nov 1; 7(11):1273-94.

18. Thorburn A, Debnath J. Targeting chaperone-mediated autophagy in cancer. Sci Transl Med. (Invited commentary). 2011 Nov 16; 3(109):109ps45.

19. Klionsky DJ et. al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. (Multi-author consortium) Autophagy. 2012 Apr;8(4): 445-544.

20. Murrow, L, Debnath, J. Autophagy as a Stress Response and Quality Control Mechanism: Implications For Cell Injury and Human Disease. Annu Rev Pathol. (Invited Review), 2013, 8: 105-37.

21. Starobinets, H, Debnath, J. Cancer, a suppression switch, Nature (News and Views, Invited Commentary), 2013, 504(7479):225-6.

22. Goldsmith, J, Levine, B., Debnath, J. Autophagy and cancer metabolism, Methods Enzymol. (Invited Review), 2014; 542:25-57.

23. Leidal, A, Debnath, J "Doubling down" on the autophagy pathway to suppress tumor growth. Genes and Development (Perspectives Articles, Invited Commentary), 2014; 28(11): 1137-9.

24. Amaravadi, R, Debnath, J. Mouse models address key concerns regarding autophagy inhibition in cancer. Cancer Discovery (Invited Commentary), 2014, 4: 873-75

Books and Chapters

1. J. Debnath and C. Fung. The Dual Roles For Autophagy in Cell Death and Survival, First Ed. Editor(s): V. Deretic. Autophagy in Infection and Immunity:Wiley-VCH, Germany. 2006.

2. M.S. Sosa, P. Bragado, J. Debnath,* J. A. Aguirre-Ghiso*. Regulation of Tumor Cell Dormancy By Tissue Microenvironments and Autophagy. 2013, Adv. Exp. Med. Biol, 734: 73-89. ***co-senior author.**

RESEARCH PROGRAM

Autophagy, adhesion independent survival, and oncogenic transformation: Integrin-mediated cell adhesion to ECM is critical for normal epithelial cell survival; in fact, ECM-deprived normal cells (unlike cancer cells) undergo apoptosis, termed anoikis. Constitutive growth factor pathway activation is a common mechanism utilized by cancer cells to evade anoikis. However, we discovered that another mechanism protects epithelial cells during anoikis-autophagy. Autophagy is a tightly regulated self-digestion process that promotes cell survival during starvation and stress. In recent follow-up studies, we have found that autophagy is robustly induced in detached cells expressing oncogenes that activate either PI3K/Akt or Ras/MAPK, and have discovered that autophagy facilitates glycolytic metabolism and proliferation during adhesion independent transformation driven by oncogenic Ras. Based on these findings, we hypothesize that autophagy uniquely contributes to the fitness of oncogene-transformed cells, allowing them to survive and expand in the absence of cell-ECM contact.

Role of autophagy on cancer metastases in vivo: In the upcoming years, my laboratory will delineate how autophagy impacts on cancer progression in mouse cancer models. We have created mice containing conditional null mutant alleles of ATG12, allowing us to delete this gene in a tissue specific manner. We are now crossing with established mouse models of breast cancer (e.g. PyMT) to define the role of autophagy in cancer progression in vivo. Our studies will focus on dissecting the requirements for autophagy as breast cancer cells disseminate into the systemic circulation, lie dormant for extended periods, and ultimately, metastasize to foreign tissue sites.

Biology and biochemistry of ATG12 and ATG12-ATG3: Ubiquitin-like protein conjugations (UBLs) are post-translational modifications that play regulatory roles in all cellular processes. Among these molecules, ATG12 is a UBL required for autophagy. Unlike other UBLs, ATG12 has been proposed to have only own known conjugation target, another autophagy gene called ATG5; indeed, this has been prevailing view for the last eleven years. However, we recently discovered that ATG12 modifies multiple protein targets and identified ATG3 as a novel substrate for ATG12 conjugation. ATG3 is the E2-like enzyme necessary for ATG8/LC3 lipidation during autophagy. ATG12-ATG3 complex formation requires ATG7 as the E1 enzyme and ATG3 autocatalytic activity as the E2, resulting in the covalent linkage of ATG12 onto a single lysine on ATG3. Surprisingly, disrupting ATG12 conjugation to ATG3 does not affect autophagy. Rather, the lack of ATG12-ATG3 complex formation produces an expansion in mitochondrial mass and inhibits cell death mediated by mitochondrial pathways. Overall, these data reveal a previously unknown role for ATG12-ATG3 in mitochondria, and implicate the ATG12

conjugation system in novel cellular functions distinct from the early steps of autophagosome formation. These results also support our broader hypothesis that ATG12 covalently modifies multiple targets within eukaryotic cells, and that these modified targets of "12-ylation" are required for not just for autophagy, but a diverse array of additional cellular processes.

SIGNIFICANT PUBLICATIONS

1. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy dependent production of secreted factors facilitates oncogenic RAS-driven invasion. Cancer Discov. 2014 Feb 10

Significance: This paper delineates a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion; an intact autophagy pathway is required for the elaboration of multiple secreted factors favoring invasion, including the proinflammatory cytokine IL6.

Role: Senior author. Becky Lock, a PhD graduate from my laboratory, and I designed the overall project. We received major input on experiments from Candia Kenific, a BMS graduate student, and Andrew Leidal, a post-doc in my laboratory. All of the authors designed and carried out the major experiments in the paper. Becky wrote the first draft of the paper, which I edited, with input from the other co-authors, to create the final version.

2. Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J, Passegué E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. Nature. 2013 Feb 21; 494(7437):323-7.

Significance: The paper demonstrates for the first time that the autophagy pathway serves as an important survival mechanism in both young and old hematopoietic stem cells (HSCs) in response to nutrient starvation and metabolic stress.

Role: Co-author. This was close collaboration with the Passague lab over the last three years. I conceived and designed the overall project with Matt Warr, post-doctoral fellow, and Emmannuelle Passague, UCSF Associate Professor of Medicine. Matt and I developed and optimized the assays to monitor autophagy in mouse hematopoietic stem cells. My lab also generated the Atg12 conditional knockout critical for these studies. Matt was the primary individual that carried out the experiments. Matt, Emmanuelle, and myself analyzed the results and wrote the paper.

3. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell. 2011 Jan 15; 22(2):165-78.

Significance: This paper demonstrates an unexpected connection between autophagy and glycolysis that facilitates adhesion-independent transformation driven by a strong oncogenic insult-mutant Ras. Inhibiting autophagy due to the genetic deletion or RNAi-mediated depletion of multiple autophagy regulators (ATGs) attenuates Ras-mediated adhesion-independent

transformation and proliferation, as well as reduces glycolytic capacity. Overall, these results point to a unique mechanism by which autophagy may promote Ras-driven tumor growth in specific metabolic contexts.

Role: Senior author. Becky Lock, UCSF BMS graduate student in my laboratory, and I designed the overall project, with major input on the glycolysis studies from Srirupa Roy, a post-doctoral fellow in my laboratory, and Sabrina Ronen, UCSF Professor of Radiology. Becky and Srirupa designed and carried out the major experiments in the paper, with assistance from the other co-authors. Becky wrote the first draft of the paper, which I edited, with input from the other co-authors, to create the final version.

4. Gupta A, Roy S, Lazar AJ, Wang WL, McAuliffe JC, Reynoso D, McMahon J, Taguchi T, Floris G, Debiec-Rychter M, Schoffski P, Trent JA, Debnath J, Rubin BP. Autophagy inhibition and antimalarials promote cell death in gastrointestinal stromal tumor (GIST). Proc Natl Acad Sci U S A. 2010 Aug 10; 107(32):14333-8.

Significance: This paper is the first to demonstrate autophagy as survival mechanism in gastrointestinal stromal tumors (GIST), the most common sarcoma in the GI tract. Although gastrointestinal stromal tumors (GIST) harboring activating KIT or platelet-derived growth factor receptor A (PDGFRA) mutations respond to treatment with targeted KIT/PDGFRA kinase inhibitors, such as imatinib mesylate, such treatments are not cytotoxic in GIST patients. Thus, this paper demonstrates autophagy inhibition as a new therapeutic target for these cancers and creates an entirely new paradigm for GIST treatment, which do not respond to traditional cytotoxic therapies.

Role: Co-senior author. This was an equal collaboration between my laboratory at UCSF and that of Dr. Brian Rubin (Cleveland Clinic), a sarcoma pathologist/biologist. Brian and I designed the overall project and the experiments, along with two post-docs-Anu Gupta (Rubin lab) and Srirupa Roy (Debnath Lab). Drs. Gupta and Roy carried out all of the experimental assays in the paper. The remaining co-authors provided tissue samples, in vivo methods, and analytical tools critical for the research project. Dr. Rubin and I wrote the paper.

5. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, Debnath J. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell. 2010 Aug 20; 142(4):590-600.

Significance: This paper challenges a long-held view in the autophagy field that ATG12, an ubiquitin-like modifier required for autophagy, possesses a single substrate, called ATG5. This paper uncovers that ATG12 is conjugated to ATG3, another enzyme required for autophagy. As individual proteins, both ATG12 and ATG3 are essential for early autophagosome formation. In contrast, the ATG12-ATG3 protein complex dramatically alters the mitochondrial network and the response to mitochondrial cell death. Overall, these results unveil a previously unrecognized role for ATG12-ATG3 in mitochondrial homeostasis, and implicate the ATG12 conjugation system in cellular functions distinct from the early steps of autophagosome formation.

Role: Senior author. Lilly Radoshevich, a UCSF graduate student in my laboratory, and I designed the overall project and all of the individual experiments in the paper. Lilly and the other co-authors carried out the experiments. Lilly wrote the first draft of the paper, which I edited to create the final version.

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RESEARCH ARTICLE

Autophagy-Dependent Production of Secreted Factors Facilitates Oncogenic RAS-Driven Invasion

Rebecca Lock, Candia M. Kenific, Andrew M. Leidal, Eduardo Salas, and Jayanta Debnath

ABSTRACT The tumor-promoting functions of autophagy are primarily attributed to its ability to promote cancer cell survival. However, emerging evidence suggests that autophagy plays other roles during tumorigenesis. Here, we uncover that autophagy promotes oncogenic RAS-driven invasion. In epithelial cells transformed with oncogenic RAS, depletion of autophagy-related genes suppresses invasion in three-dimensional culture, decreases cell motility, and reduces pulmonary metastases *in vivo*. Treatment with conditioned media from autophagy-competent cells rescues the invasive capacity of autophagy-deficient cells, indicating that these cells fail to secrete factors required for RAS-driven invasion. Reduced autophagy diminishes the secretion of the promigratory cytokine interleukin-6 (IL-6), which is necessary to restore invasion of autophagy-deficient cells. Moreover, autophagy-deficient cells exhibit reduced levels of matrix metalloproteinase 2 and WNT5A. These results support a previously unrecognized function for autophagy in promoting cancer cell invasion via the coordinate production of multiple secreted factors.

SIGNIFICANCE: Our results delineate a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion. We demonstrate that an intact autophagy pathway is required for the elaboration of multiple secreted factors favoring invasion, including IL-6. *Cancer Discov; 4(4); 466–79. ©2014 AACR.*

INTRODUCTION

The RAS proteins are members of a family of small GTPases critical in mediating cellular responses following activation by upstream extracellular signals, such as growth factors. Oncogenic mutations in RAS, which result in constitutive activation, are found in approximately 30% of human cancers; they are highly prevalent in several carcinomas, including lung, pancreas, and colon (1, 2). Notably, oncogenic RAS drives diverse cellular programs—proliferation, cell survival, migration, invasion, and alterations in differentiation—that support tumor initiation and progression. Such mutations present a formidable therapeutic obstacle, because patients harboring mutant KRAS are refractory to most available systemic therapies and exhibit extremely poor survival (2). Hence, identifying new processes to target cancer cells with

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hyperactive RAS remains a question of immense clinical significance. One such pathway may be macroautophagy (autophagy), a tightly controlled lysosomal degradation process that promotes cell survival during nutrient starvation and stress. Recent evidence indicates that basal autophagy levels are enhanced upon oncogenic RAS activation and support RAS-driven transformation and tumorigenesis (3–7).

The tumor-promoting functions of autophagy are largely ascribed to its importance as a survival pathway in response to diverse environmental stresses (8, 9). For example, enhanced autophagy is observed in poorly perfused, hypoxic tumor regions, and loss of autophagy is associated with increased necrosis (10). Autophagy also promotes tumor cell survival in response to various cytotoxic and targeted chemotherapies (11). Importantly, studies of oncogenic RAS transformation have revealed that the protumor effects of autophagy are not limited to increased survival of cancer cells under duress; rather, autophagy contributes to the metabolic fitness of the entire tumor population (3–6). Because strong oncogenic insults, such as RAS activation, are marked by profound metabolic alterations that drive both energy production and biosynthetic capacity in rapidly proliferating cells, it has been hypothesized that autophagy maintains key metabolic pathways in RAS-transformed cells. In support, a growing body of work has unveiled a requirement for autophagy in driving proliferation as well as sustaining multiple core metabolic functions in RAS-transformed cells (3–7). These results are not unique to oncogenic RAS activation, as deletion of *RB1CC1/FIP200*, a mediator of autophagosome initiation, inhibits polyoma middle T–driven mammary cancer, due to reduced proliferation and glucose metabolism (12).

In addition to its effects on proliferation and metabolism, oncogenic RAS drives diverse aggressive cellular behaviors that support tumor progression and metastasis; importantly, RAS-transformed epithelial cells exhibit highly invasive behavior associated with an epithelial-to-mesenchymal transition (EMT; ref. 13). Here, in epithelial cells transformed with oncogenic RAS, we demonstrate that autophagy facilitates extracellular matrix (ECM) invasion, tumor cell motility, and pulmonary metastasis *in vivo*. Using a three-dimensional (3D) culture system, we uncover that autophagy inhibition restricts RAS-driven cell invasion and restores several aspects of normal epithelial architecture, including the polarized deposition of basement membrane and cell–cell junctional integrity. Furthermore, autophagy is required for the production of multiple secreted factors in RAS-transformed cells, including interleukin-6 (IL-6), matrix metalloproteinase 2 (MMP2), and WNT5A, which altogether facilitate cancer cell invasion.

RESULTS

Autophagy Promotes Invasion Driven by Oncogenic RAS in 3D Culture

To elucidate how autophagy affects the cellular behavior of RAS-transformed epithelial cells, we used the MCF10A 3D epithelial culture system to interrogate how autophagy affects the growth and morphogenesis of cells expressing oncogenic RAS (14). We generated stable pools of MCF10A human mammary epithelial cells expressing a control vector (BABE) or an oncogenic form of HRAS ($HRAS^{V12}$) that enhances basal autophagy and elicits robust anchorage-independent transformation (3). When cultured on laminin-rich ECM, control MCF10A cells formed hollow, spherical acini (Supplementary Fig. S1A; ref. 15). In contrast, $HRAS^{V12}$-transformed cells produced grossly aberrant structures notable for extensive protrusions that invaded the surrounding ECM. Individual $HRAS^{V12}$ structures formed these invasive protrusions in as early as 3 to 5 days, ultimately producing disorganized networks of cells intermingled with large cell clusters after 8 days in 3D culture (Fig. 1A and B, left). The 3D morphology we observed using $HRAS^{V12}$ MCF10A cells resembles that reported for mouse mammary cells expressing oncogenic RAS and grown in a 3D collagen matrix (16).

To inhibit autophagy in this experimental system, we stably expressed unique short hairpin RNAs (shRNA) against two autophagy genes (ATG)—*ATG7* (sh*ATG7*-1 and sh*ATG7*-2) or *ATG12* (sh*ATG12*)—in MCF10A cells expressing $HRAS^{V12}$. ATG7 or ATG12 knockdown decreased target protein levels, reduced basal- and starvation-induced autophagy in Hank's Buffered Saline Solution (HBSS), and increased protein levels of the autophagy substrate p62/SQSTM1 (Supplementary Fig. S1B–S1E). In 3D culture, the invasive protrusions observed with oncogenic RAS activation were profoundly attenuated in ATG-deficient cells. Instead, $HRAS^{V12}$ sh*ATG* (shRNAs against autophagy genes) structures were spherical in morphology, similar to nontransformed BABE controls (Fig. 1A and B). Decreased invasive protrusions following autophagy inhibition were also observed upon stable *ATG3* knockdown (sh*ATG3*), and upon treatment with chloroquine or bafilomycin A, two lysosomal inhibitors that block the late steps of autophagy (Supplementary Fig. S1F). Importantly, ATG knockdown in $HRAS^{V12}$ cells did not affect RAS expression– or activation–associated phosphorylation of the major downstream effector MAPK–ERK (Supplementary Fig. S1G). Thus, the reduction in 3D invasive protrusions following ATG knockdown is not due to decreased expression or activity of oncogenic RAS.

The disruption of basement membrane integrity is a hallmark of carcinoma invasion *in vivo* (14). To corroborate whether the protrusions we observed in $HRAS^{V12}$-transformed 3D cultures represented invasive behavior, we first evaluated basement membrane integrity by examining the expression and localization of the basement membrane protein LAMA5 (laminin 5) in $HRAS^{V12}$-derived acini. Consistent with previous reports, control (BABE) nontransformed MCF10A acini displayed polarized deposition of LAMA5 onto the basal surface (Fig. 2A, left; ref. 15). In contrast, the expression of $HRAS^{V12}$ resulted in cytosolic accumulation of LAMA5, with no evidence of polarized deposition at the cell–ECM interface. Notably, this aberrant cytosolic staining pattern was especially prominent in the protrusions of $HRAS^{V12}$ cultures. Correlating with the decreased formation of invasive protrusions, ATG knockdown restored polarized LAMA5 secretion; based on this marker, most individual structures in ATG-deficient $HRAS^{V12}$ cultures were encompassed by an intact basement membrane (Fig. 2A). Hence, in addition to restricting the formation of invasive protrusions, autophagy inhibition restored

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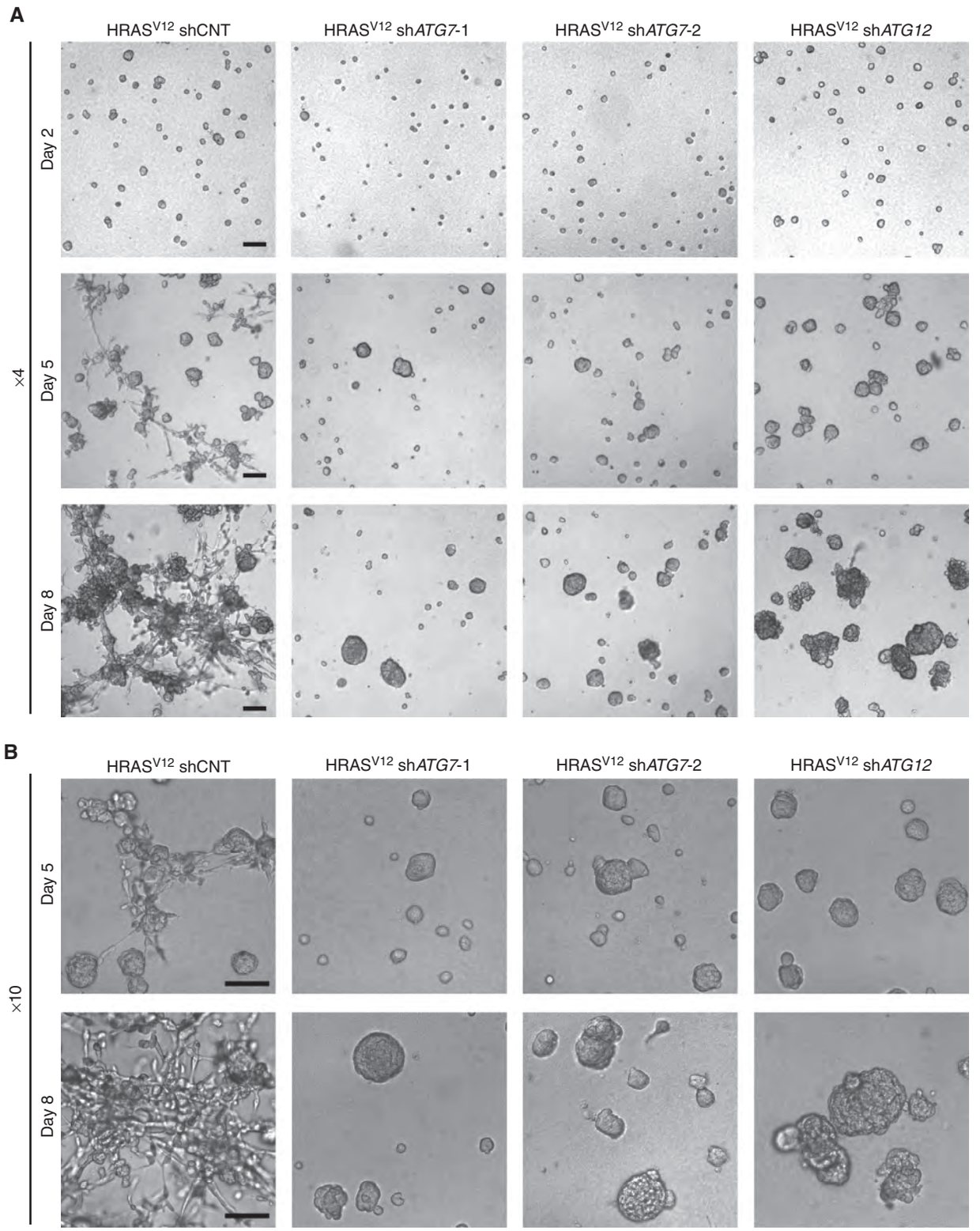

Figure 1. Autophagy is required for the formation of invasive protrusions mediated by HRAS^{V12} in 3D culture. **A** and **B,** HRAS^{V12} MCF10A cells stably expressing shCNT or sh*ATG*s were 3D cultured on Matrigel for the indicated number of days. Representative phase contrast images at the indicated magnifications are shown. Scale bar, 100 μm.

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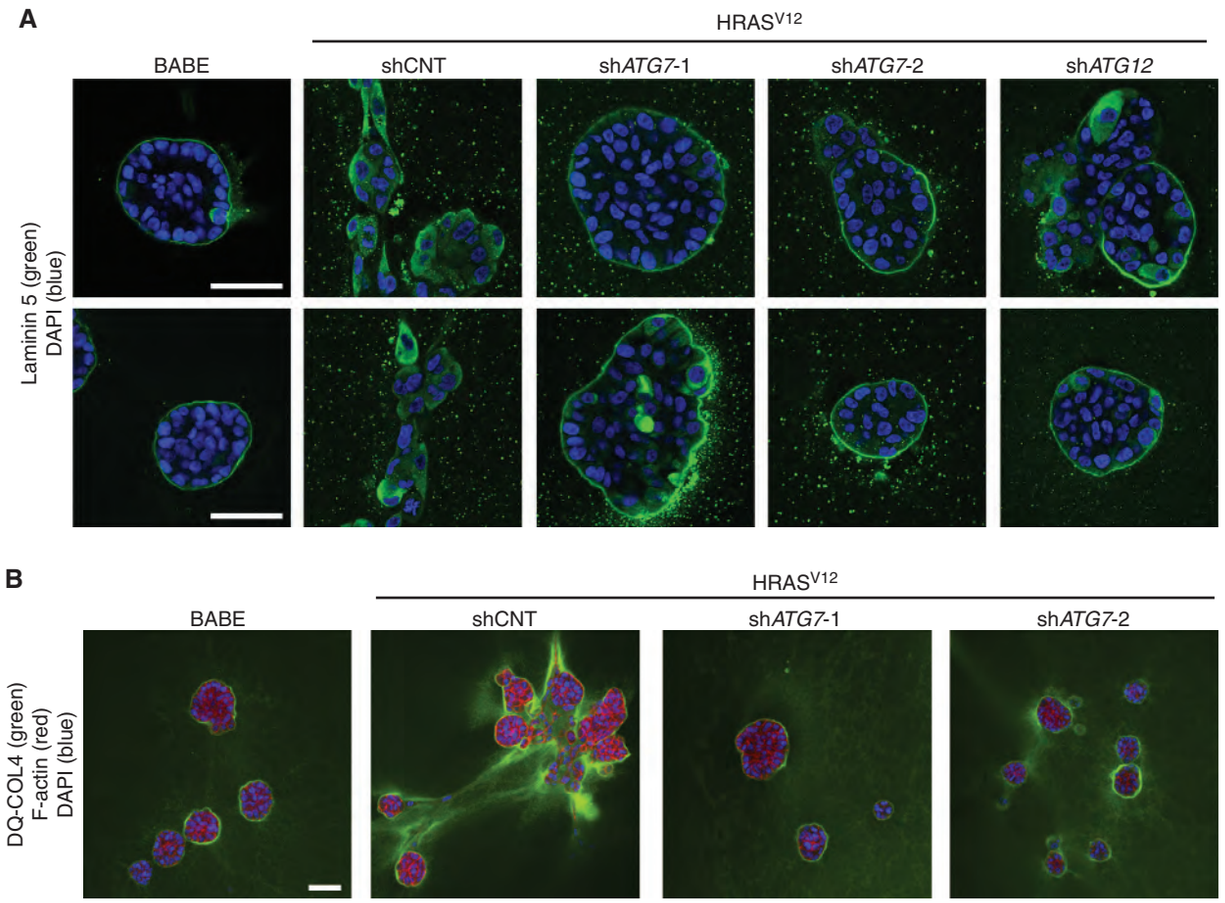

Figure 2. Autophagy inhibition in HRAS^{V12} cells restores basement membrane integrity and restricts ECM proteolysis in 3D culture. **A,** HRAS^{V12} cells expressing shCNT or sh*ATG*s were 3D cultured on Matrigel for 8 days. Structures were fixed and immunostained with antibodies against the basement membrane protein LAMA5 (human specific), counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to detect nuclei, and imaged by confocal microscopy. Two representative images of each condition are shown. Scale bar, 50 μm. **B,** HRAS^{V12} MCF10A cells were 3D cultured on Matrigel containing 25 μg/mL fluorescein DQ-COL4 for 5 days. Structures were fixed, counterstained with phalloidin (to visualize F-actin) and DAPI, and imaged by confocal microscopy. Green fluorescence represents areas of proteolytic cleavage of the DQ-COL4 present in the ECM. Scale bar, 50 μm.

polarized basement membrane secretion typically absent in HRAS^{V12} shCNT (nontargeting control shRNA) structures.

To extend these results, we evaluated ECM proteolytic activity in control and autophagy-deficient HRAS^{V12} cultures by assessing fluorescence emanating from the proteolytic cleavage of dye-quenched collagen IV (DQ-COL4). In control (BABE) nontransformed acini, we observed a faint ring of fluorescence surrounding each structure, corresponding to COL4 degradation due to the normal outgrowth of acini during 3D morphogenesis. On the other hand, HRAS^{V12} shCNT-expressing structures exhibited high levels of fluorescence that extended well beyond the immediate vicinity of individual structures (Fig. 2B). Notably, streaks of fluorescence connecting adjacent structures were frequently observed in HRAS^{V12} shCNT cultures (Fig. 2B), which resembled the networks of invasive protrusions (Fig. 1B). In contrast, HRAS^{V12} sh*ATG*-derived structures exhibited a ring-like COL4 degradation pattern that was restricted to the cell–ECM interface, similar to that observed in nontransformed controls (Fig. 2B). Thus, the absence of morphologic protrusions in ATG-deficient HRAS^{V12} cultures was associated with the restoration of basement membrane integrity and reduced ECM proteolytic activity. Together, these findings corroborate that autophagy supports RAS-driven invasion in 3D culture.

ATG Depletion in HRAS^{V12} Structures Does Not Promote Apoptosis or Proliferation Arrest in 3D Culture

We next evaluated the impact of autophagy inhibition on oncogenic RAS-driven proliferation and cell survival. During normal MCF10A acinar morphogenesis, autophagy inhibition results in the enhanced apoptosis of cells occupying the luminal space (17). To test whether autophagy deficiency similarly affected apoptosis in HRAS^{V12} structures, we immunostained structures with an antibody against cleaved CASP3 (caspase-3). In contrast to the robust luminal apoptosis observed in control acini (BABE), only isolated cleaved CASP3-positive cells were observed in HRAS^{V12} shCNT structures, consistent with the ability of oncogenic RAS to promote cell survival in 3D culture (Fig. 3A).

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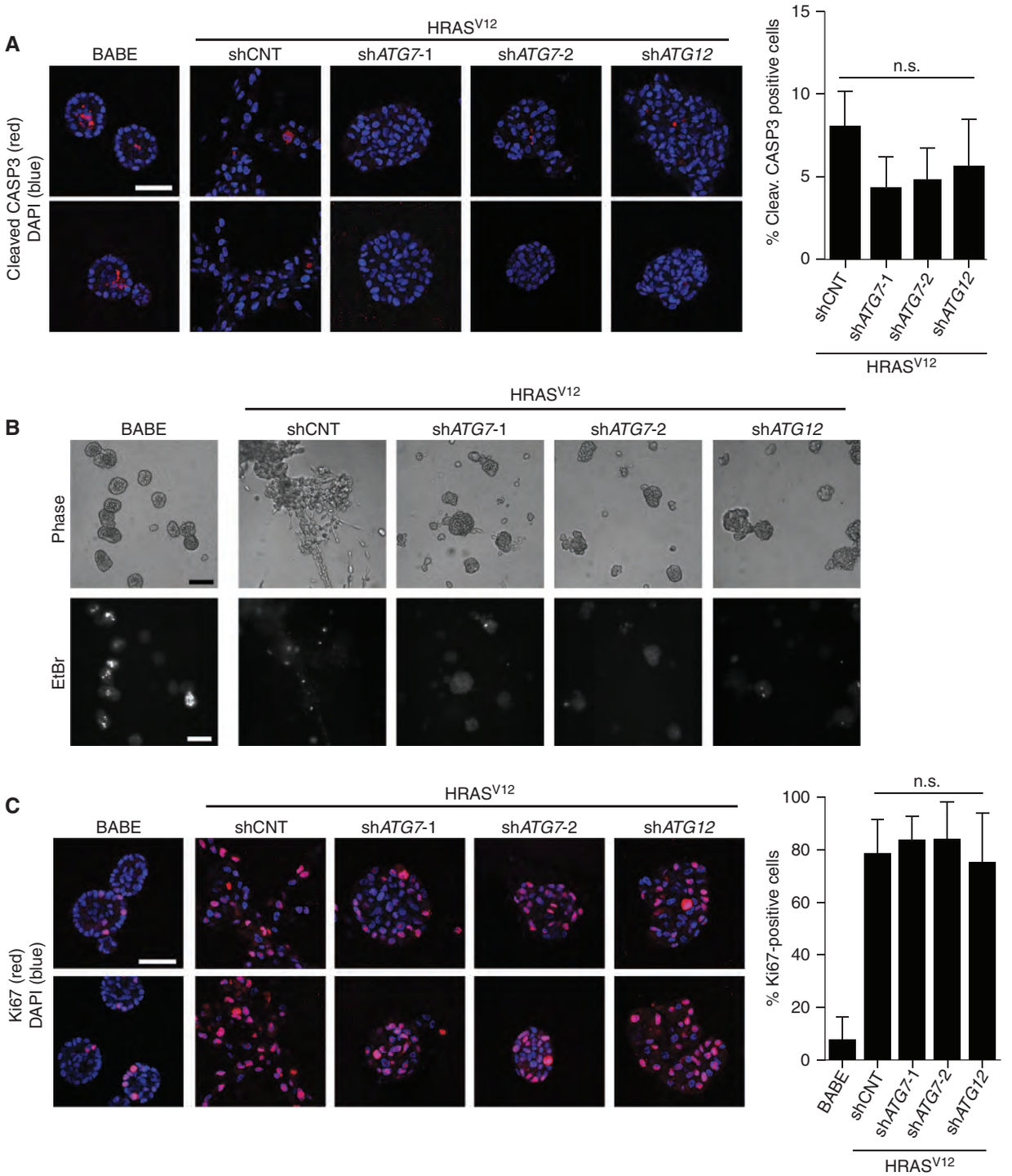

Figure 3. Autophagy inhibition in HRAS^{V12} MCF10A structures does not promote apoptosis or proliferation arrest. **A,** left, two representative images of day 8 3D cultures of BABE and HRAS^{V12} MCF10A cells expressing shCNT or sh*ATG*s immunostained with an antibody against cleaved CASP3 and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to detect nuclei. Scale bar, 50 μm. Right, quantification of cleaved CASP3-positive cells present within 3D cultures of each indicated cell type (mean ± SD, Student *t* test). **B,** representative phase (top) and corresponding wide-field fluorescence (bottom) images of BABE and HRAS^{V12} cells expressing shCNT or sh*ATG*s stained with the intravital dye EtBr. Scale bar, 100 μm. **C,** left, two representative images of day 8 3D cultures of BABE and HRAS^{V12} cells expressing shCNT or sh*ATG*s immunostained with an antibody against Ki67 and DAPI counterstained. Scale bar, 50 μm. Right, quantification of Ki67-positive nuclei present within 3D cultures of each indicated cell type (mean ± SD, Student *t* test).

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Upon enumerating cleaved CASP3-positive cells from these 3D cultures, we found that ATG knockdown did not significantly affect apoptosis in comparison with shCNT cultures (Fig. 3A). To assess whether autophagy inhibition potentially affected nonapoptotic death processes, we also stained day 8 3D cultures with ethidium bromide (EtBr), an intravital dye that is incorporated into all dying cells. Although acini derived from nontransformed (BABE) cells displayed high levels of EtBr staining corresponding to luminal cell death (Fig. 3B), HRAS^{V12} structures displayed only occasional EtBr cells scattered throughout the structures. Although ATG knockdown in HRAS^{V12} cultures resulted in spherical structures that lacked invasive protrusions, we did not observe any increase in EtBr staining in these cultures (Fig. 3B). Thus, in contrast to normal and oncogenic PIK3CA MCF10A acinar morphogenesis, autophagy inhibition does not promote apoptosis in RAS-transformed 3D structures (17, 18).

To evaluate the effects of autophagy inhibition on the proliferative capacity of HRAS^{V12} structures, we immunostained cultures with the proliferation marker Ki67 on day 8, a time point at which normal MCF10A acini exhibit reduced proliferation (19). As expected, low levels of Ki67-positive cells were observed in BABE structures (Fig. 3C, left). However, both control and autophagy-deficient HRAS^{V12} structures displayed high levels of Ki67-positive cells (Fig. 3C). Overall, these results indicate that although autophagy deficiency potently restricts HRAS^{V12}-driven invasion, it does not universally suppress the diverse oncogenic effects of HRAS^{V12} in 3D culture, including the ability of activated RAS to inhibit apoptosis and sustain proliferation.

Autophagy Supports Oncogenic RAS-Driven Cell Migration *In Vitro* and Pulmonary Metastasis *In Vivo*

Because defects in invasive capacity are often associated with diminished cell motility, we next measured cell migration in autophagy-competent and -deficient epithelial cells. Upon ATG depletion, HRAS^{V12} MCF10A cells demonstrated an approximately 30% reduction in migratory capacity in a monolayer wound-healing assay of cell migration (Fig. 4A). Similar results were obtained using a Transwell migration assay, which demonstrated a significant decrease in migration of ATG knockdown cells (Fig. 4B). We further corroborated these results using MDA-MB-231 cells, a highly migratory, *KRAS*-mutant breast cancer cell line. siRNA-mediated knockdown of either ATG7 or ATG12 in MDA-MB-231 cells resulted in reduced LC3-II formation (Supplementary Fig. S1H) as well as decreased wound closure (Fig. 4C, left). A similar decrease in MDA-MB-231 migration was also observed in the presence of the lysosomal inhibitor bafilomycin A (Fig. 4C, right). Therefore, in addition to supporting invasion of HRAS^{V12} MCF10A cells in 3D culture, autophagy facilitates the migration of cells expressing oncogenic RAS in monolayer culture. Finally, we used an experimental metastasis assay to evaluate whether the effects of autophagy inhibition on invasion and migration correlated with changes in metastatic capacity *in vivo*; in support, the ability of HRAS^{V12} MCF10A cells to produce pulmonary metastases was reduced upon ATG knockdown (Fig. 4D).

Altered Differentiation of HRAS^{V12} MCF10A Cells upon Autophagy Inhibition

Constitutive RAS activation alters epithelial differentiation by driving an EMT (20, 21), a process associated with increased invasive and migratory capacity *in vitro* and with metastatic capacity *in vivo* (13). Therefore, we evaluated how autophagy inhibition affects protein expression changes associated with RAS-induced EMT. We isolated BABE-, HRAS^{V12} shCNT-, and HRAS^{V12} sh*ATG*-expressing cells from day 8 3D cultures and determined the protein expression of a panel of EMT-associated genes by immunoblotting. In comparison with nontransformed BABE acini, HRAS^{V12} shCNT structures displayed decreased keratin 14 (KRT14), an epithelial marker, and a corresponding increase in the mesenchymal protein vimentin (VIM; Supplementary Fig. S2A). ATG knockdown reversed these HRAS^{V12}-driven changes in differentiation, resulting in an increase in KRT14 protein levels and a corresponding decrease in VIM levels compared with HRAS^{V12} shCNT cells isolated from 3D culture (Supplementary Fig. S2A). However, autophagy inhibition had minimal effects on other EMT markers that were altered by oncogenic RAS expression. Only a slight increase in E-cadherin (CDH1) was observed in sh*ATG* cells, decreased fibronectin (FN1) was only observed in sh*ATG7*-1–expressing cells, and N-cadherin (CDH2) levels were unchanged following ATG knockdown (Supplementary Fig. S2A).

During EMT, cells commonly lose the ability to form cell–cell junctions (22). Therefore, we analyzed the effects of autophagy inhibition on cell–cell junctional integrity in HRAS^{V12} 3D structures by immunostaining for β-catenin (CTNNB1). Normal MCF10A acini (BABE) displayed strong β-catenin staining at cell–cell contacts, indicating intact adherens junctions, whereas the expression of HRAS^{V12} resulted in a near-complete loss of β-catenin junctional staining; in these cultures, only isolated focal areas of junctional β-catenin staining were observed (Supplementary Fig. S2B). Upon ATG knockdown in HRAS^{V12} structures, both the expression and junctional localization of β-catenin were significantly restored (Supplementary Fig. S2B). On the basis of these results, we conclude that autophagy inhibition modulates certain aspects of mesenchymal differentiation in RAS-transformed cells in 3D culture, most notably the suppression of VIM, as well as the restoration of KRT14 expression and epithelial cell–cell contacts. Nonetheless, autophagy deficiency does not broadly suppress RAS-driven EMT.

ATG Knockdown in HRAS^{V12} Cells Inhibits the Production of Proinvasive Secreted Factors in 3D Culture

Cell migration and invasion involves the secretion of multiple factors that cooperate to promote motility and to degrade the surrounding ECM (23, 24). To ascertain whether defects in RAS-driven invasion observed following autophagy suppression were the result of decreased production of proinvasive factors, we performed a coculture assay in which HRAS^{V12} sh*ATG7*-1 cells (coexpressing GFP for tracking purposes) were combined with HRAS^{V12} shCNT cells at a ratio of 3:1, respectively. Although HRAS^{V12} sh*ATG7*-1–GFP cells cultured alone grew as spherical structures (Fig. 5A, left), upon coculture with HRAS^{V12} shCNT cells, HRAS^{V12} sh*ATG7*-1–GFP structures became dispersed and formed invasive protrusions

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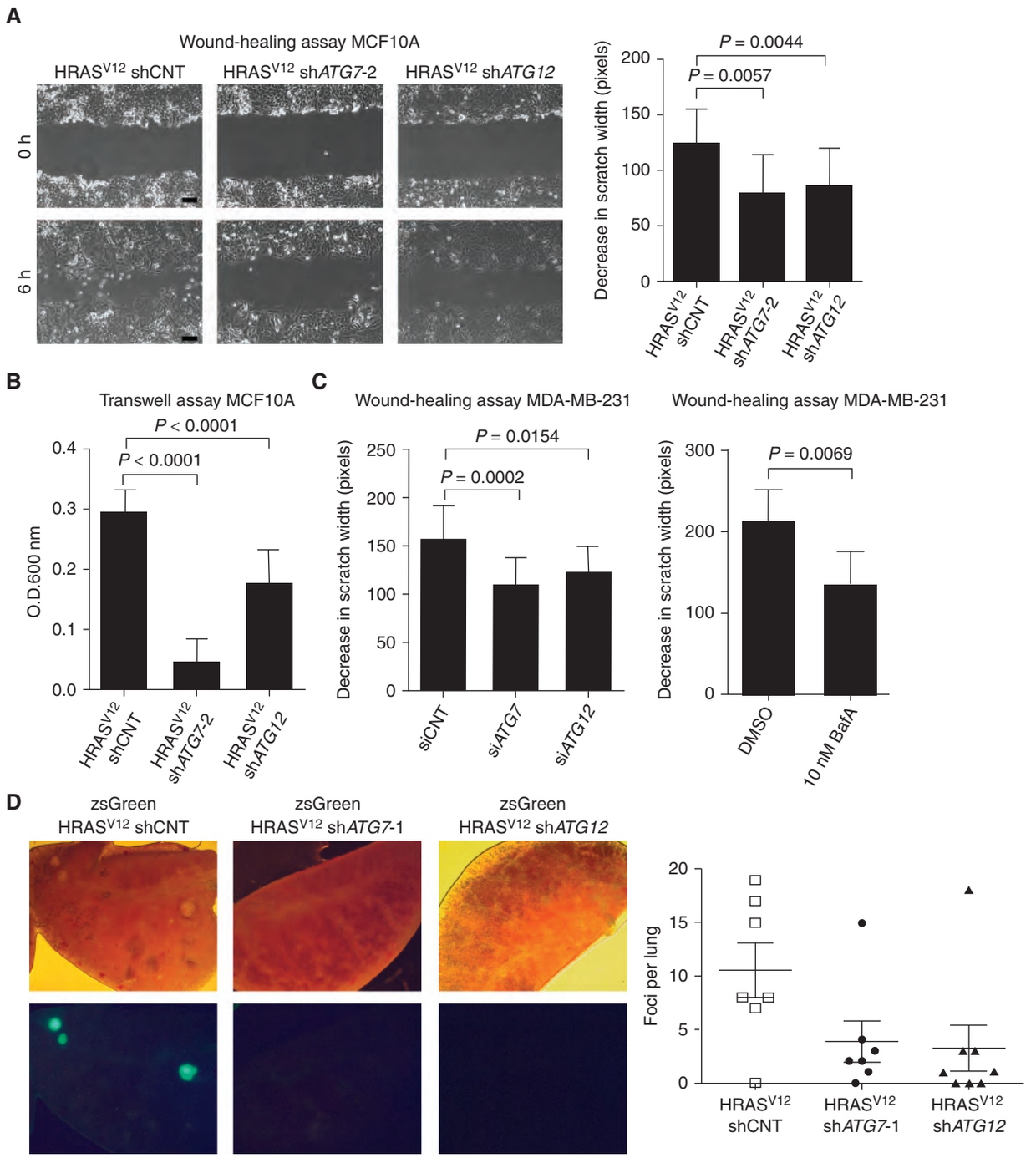

Figure 4. ATG knockdown suppresses the motility and reduces the metastatic potential of cells expressing oncogenic RAS. **A,** representative images (left) and quantification (right) of wound-healing assay on HRAS^{V12} MCF10A cells expressing shCNT or sh*ATG*s. Confluent monolayers were scratched and wound width was measured at 0 and 6 hours after initial wounding to quantify the decrease in scratch width (mean ± SD, Student *t* test; shCNT, $n = 16$; sh*ATG7*-2, $n = 8$; sh*ATG12*, $n = 14$). Scale bar, 100 μm. **B,** Transwell migration of HRAS^{V12} MCF10A cells expressing shCNT or sh*ATG*s. Twenty-four hours after plating, cells that migrated to the bottom of the filter were stained with crystal violet. Results are expressed as the mean crystal violet extracted from stained cells (mean ± SD, Student *t* test, $n = 9$). **C,** wound-healing assays of MDA-MB-231 cells expressing si*ATG*s or in the presence of 10 nmol/L bafilomycin A (BafA). Graphs represent the decrease in scratch width at 10 and 9 hours after initial wounding, respectively [mean ± SD, Student *t* test; siCNT, $n = 16$; si*ATG7*, $n = 16$; si*ATG12*, $n = 10$; dimethyl sulfoxide (DMSO), $n = 6$; BafA, $n = 6$]. **D,** representative images (left) and quantification (right) of ZsGreen-positive metastatic foci following tail vein injection of ZsGreen-expressing HRAS^{V12} shCNT, sh*ATG7*-1, or sh*ATG12* cells (mean ± SEM; shCNT, $n = 7$; sh*ATG7*-1, $n = 7$; sh*ATG12*, n = 8).

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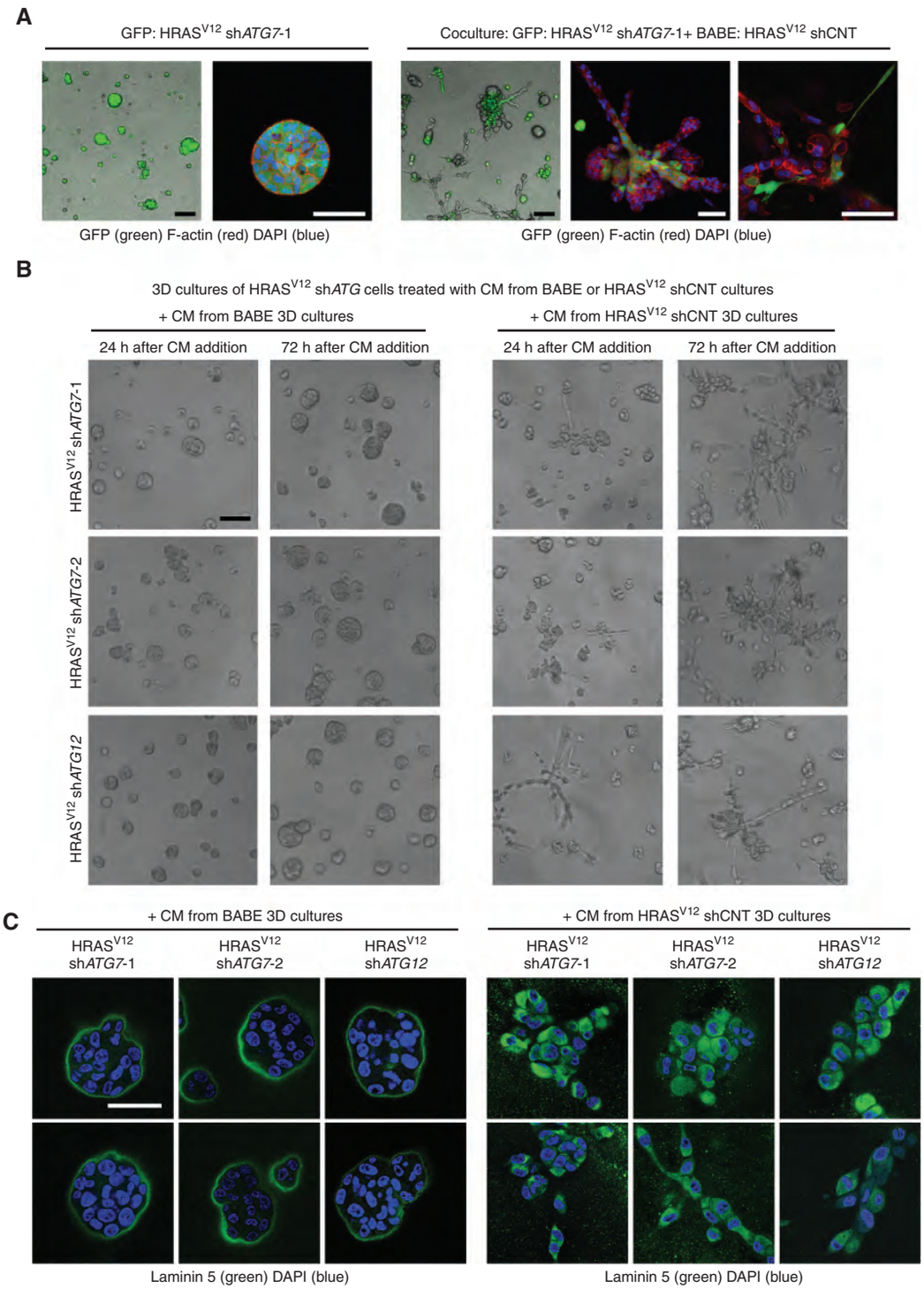

Figure 5. ATG knockdown in HRAS^{V12} cells inhibits the production of proinvasive secreted factors in 3D culture. **A,** 3D coculture of HRAS^{V12} sh*ATG7*-1 with HRAS^{V12} shCNT cells rescues invasion of HRAS^{V12} sh*ATG7*-1 cells. HRAS^{V12} sh*ATG7*-1 cells expressing GFP were cultured for 8 days in 3D either alone (left) or together with HRAS^{V12} shCNT cells expressing an empty vector (BABE). Structures were imaged by phase contrast and wide-field fluorescence microscopy or fixed, counterstained with phalloidin (to visualize F-actin) and 4′,6-diamidino-2-phenylindole (DAPI), and imaged by confocal microscopy. Phase: scale bar, 100 μm. Confocal: scale bar, 50 μm. **B,** HRAS^{V12} MCF10A cells expressing sh*ATG*s were cultured in 3D for 3 days and subsequently treated with BABE or HRAS^{V12} shCNT conditioned media (CM). Representative phase contrast images at 24 and 72 hours following the addition of conditioned media. Scale bar, 100 μm. **C,** 3D cultures of HRAS^{V12} MCF10A cells expressing sh*ATG*s were treated with BABE or HRAS^{V12} shCNT conditioned media for 72 hours; thereafter, cultures were fixed and immunostained with an antibody against LAMA5 (human specific) to detect basement membrane and DAPI counterstained. Two representative images per condition are shown. Scale bar, 50 μm.

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(Fig. 5A, right). Hence, we hypothesized that factors from neighboring HRAS^{V12} shCNT cells are sufficient to rescue *in trans* the invasion defect in HRAS^{V12} sh*ATG7*-1 cells. To further test this prediction, we grew HRAS^{V12} sh*ATG* cells in 3D culture for 3 days and subsequently treated these structures with conditioned media produced from either BABE or HRAS^{V12} shCNT cultures. HRAS^{V12} sh*ATG* structures remained as compact spheres following treatment with BABE conditioned media (Fig. 5B and Supplementary Fig. S3A). In contrast, conditioned medium from HRAS^{V12} shCNT cultures elicited invasive protrusions at 24 hours following treatment, which became fully evident by 72 hours (Fig. 5B and Supplementary Fig. S3A); notably, conditioned medium addition did not induce invasion in nontransformed BABE acini (Supplementary Fig. S3B). Furthermore, basement membrane integrity was lost in HRAS^{V12} sh*ATG* cells treated with HRAS^{V12} conditioned medium (Fig. 5C). These findings demonstrate that autophagy inhibition in HRAS^{V12} cells inhibits the production of secreted factors required for RAS-driven invasion in 3D culture.

Diminished Secretion of IL-6 Contributes to Reduced Invasion in Autophagy-Deficient HRAS^{V12} Cells

During RAS-induced senescence, ATG depletion inhibits IL-6 production following acute oncogenic RAS activation in IMR90 fibroblasts, indicating that autophagy supports the production of IL-6 in response to oncogenic RAS activation (25). Because IL-6 has been demonstrated to support RAS-driven tumorigenesis, promote migration, and invasion, and also drive EMT (26–28), we tested whether IL-6 levels were altered in HRAS^{V12} sh*ATG* 3D cultures. Analysis of IL-6 in conditioned media collected from 3D cultures by ELISA indicated a significant reduction in secreted IL-6 levels in HRAS^{V12} sh*ATG*-expressing cultures compared with HRAS^{V12} shCNT cultures (Fig. 6A). Furthermore, this decrease in

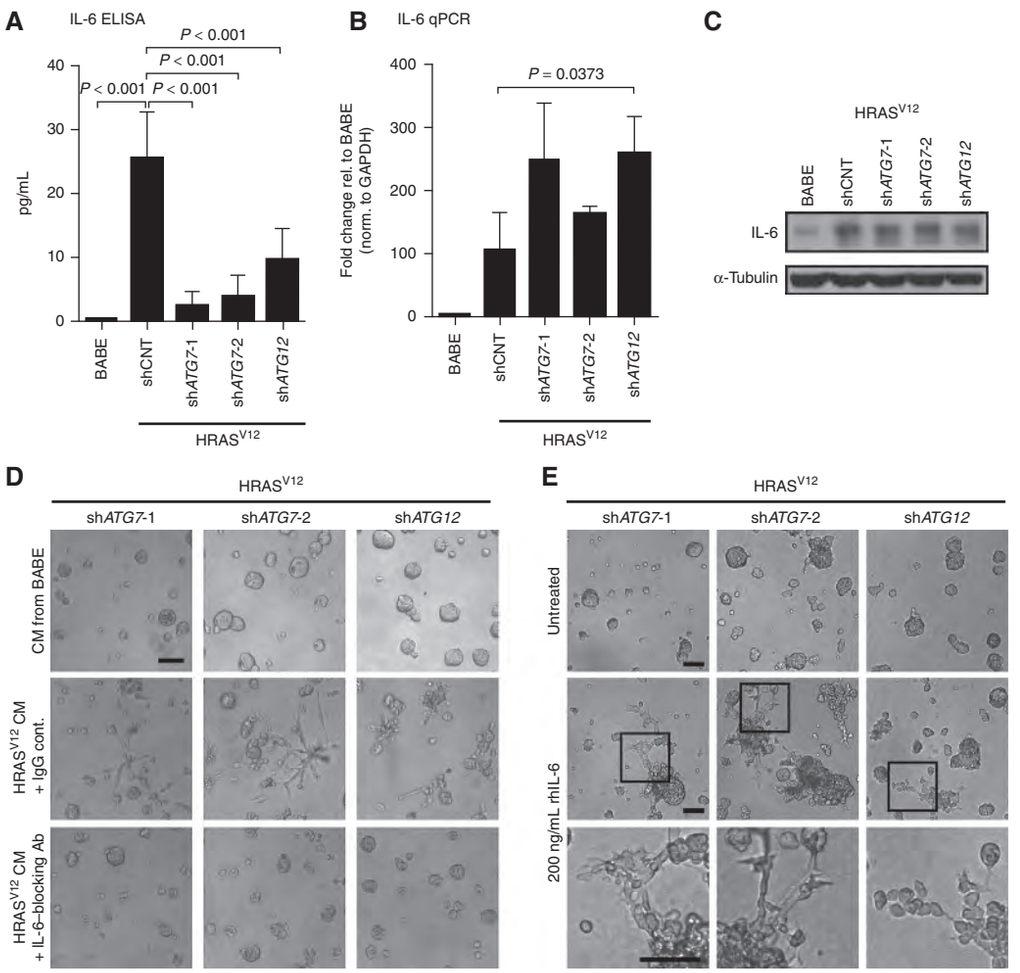

Figure 6. Autophagy supports IL-6 secretion necessary for oncogenic RAS-driven invasion in 3D culture. **A,** levels of IL-6 in conditioned media (CM) collected on day 6 from 3D cultures of the indicated cell types (mean ± SD, ANOVA; BABE, $n = 3$; HRAS^{V12}, $n = 5$). **B,** *IL6* expression levels normalized to *GAPDH* in cells collected from day 8 3D cultures (mean relative to BABE ± SD, Student *t* test, $n = 3$). qPCR, quantitative PCR. **C,** IL-6 protein levels in day 8 3D cultures from the indicated cell types. **D,** representative phase contrast images of HRAS^{V12} sh*ATG* 3D cultures treated for 48 hours with BABE conditioned media (top) or with HRAS^{V12} shCNT conditioned media containing an IL-6 function–blocking antibody (Ab; bottom) or IgG control antibody (middle). Scale bar, 100 μm. **E,** representative phase contrast images of HRAS^{V12} sh*ATG* 3D cultures grown in the presence or absence of 200 ng/mL recombinant human IL-6 (rhIL-6) for 7 days. Scale bar, 100 μm.

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secreted IL-6 was not the result of reduced *IL6* gene expression; in fact, quantitative PCR (qPCR) analysis revealed that *IL6* transcript levels in HRAS^{V12} sh*ATG* cells were increased, rather than decreased, in comparison with HRAS^{V12} shCNT cells (Fig. 6B). Notably, studies of RAS-induced senescence similarly demonstrated that autophagy-deficient cells exhibit reduced IL-6 protein levels due to impaired translation, rather than transcription (25, 29). In contrast, we uncovered that ATG depletion did not attenuate IL-6 protein levels in RAS-transformed cells grown in 3D culture (Fig. 6C). These results suggest that autophagy facilitates IL-6 secretion during HRAS^{V12} 3D morphogenesis.

To ascertain the functional significance of these results, we interrogated whether IL-6 was necessary for HRAS^{V12}-driven invasion in 3D culture. First, we treated HRAS^{V12} sh*ATG* structures with HRAS^{V12} shCNT conditioned media in the presence versus absence of an IL-6 function–blocking antibody. The addition of IL-6 function–blocking antibody attenuated the ability of HRAS^{V12} shCNT conditioned media to promote invasive protrusions in HRAS^{V12} sh*ATG* cultures, whereas an immunoglobulin G (IgG) isotype control had no effect (Fig. 6D). In parallel, we tested how exogenous recombinant human IL-6 (rhIL-6) treatment affected HRAS^{V12} sh*ATG* cells during 3D morphogenesis. rhIL-6 addition did not affect nontransformed BABE acini (Supplementary Fig. S3C) but partly restored invasion in HRAS^{V12} sh*ATG* cultures, resulting in large globular structures, increased invasive protrusions, and loss of basement membrane integrity (Fig. 6E and Supplementary S3D–S3E). Also, rhIL-6 addition partially reversed the effects of autophagy inhibition on KRT14 and VIM expression in HRAS^{V12} sh*ATG7* cells (Supplementary Fig. S3F). Hence, our results suggest that autophagy promotes efficient IL-6 secretion by HRAS^{V12} cells in 3D culture, which is necessary for invasion.

Autophagy Facilitates *MMP2* and *WNT5A* Expression by HRAS^{V12} Cells in 3D Culture

In addition to identifying a defect in IL-6 production following ATG knockdown, we performed a qPCR array to measure the expression levels of genes involved in EMT and invasion, and identified *WNT5A* and *MMP2* as two candidate factors whose expression was upregulated in HRAS^{V12} cells relative to BABE cells but potently suppressed upon autophagy inhibition. qPCR analysis of cells collected from 3D cultures confirmed a 2-fold decrease in *MMP2* and *WNT5A* expression in HRAS^{V12} sh*ATG* cells compared with HRAS^{V12} shCNT (Fig. 7A and B). Notably, we also evaluated the effects of rhIL-6 treatment on *MMP2* and *WNT5A* expression in sh*ATG7*-1 cultures and found that this was not sufficient to rescue expression, indicating that regulation of these factors was independent of IL-6 (Supplementary Fig. S3G).

Because these secreted factors have been implicated in cell migration and invasion, we further evaluated whether their decreased expression following ATG knockdown also contributed to the reduced invasive potential of HRAS^{V12} sh*ATG* cells. First, we used gelatin zymography to assess MMP2 activity in conditioned media from 3D cultures. MMP2 activity was enhanced in HRAS^{V12} cells compared with nontransformed (BABE) controls, and upon ATG knockdown in HRAS^{V12} cells, this activity was reduced (Fig. 7C). The increase in MMP2 expression and secretion following constitutive RAS activation was necessary for RAS-driven invasion, as addition of an MMP2 inhibitor, Arp-100, was sufficient to inhibit the formation of invasive protrusions in HRAS^{V12} 3D cultures (Fig. 7D). Furthermore, the decrease in *WNT5A* expression correlated with a decrease in WNT5A protein levels in HRAS^{V12} sh*ATG* cells isolated from 3D culture (Fig. 7E). Moreover, the addition of recombinant WNT5A to HRAS^{V12} sh*ATG7*-1 3D cultures promoted the dissociation of cells within the structures and enhanced the formation of invasive protrusions (Fig. 7F). Thus, in addition to IL-6, autophagy facilitates the production of multiple secreted promigratory and invasive factors that support RAS-driven invasion in 3D culture.

DISCUSSION

Our results delineate a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion and migration. Using a 3D culture system, we demonstrate that suppression of autophagy in HRAS^{V12} MCF10A cells restricts the formation of invasive protrusions, restores basement membrane integrity, and attenuates ECM proteolysis. In addition, autophagy inhibition diminishes cell migration *in vitro* and pulmonary metastasis *in vivo*. Upon treatment with conditioned media produced from autophagy-competent HRAS^{V12} cells, invasion is completely restored in autophagy-deficient HRAS^{V12} cultures, indicating that autophagy mediates the production of secreted factors that drive invasion in oncogenic cells. In further support, we uncover that autophagy inhibition elicits the coordinate reduction of multiple molecules favoring invasion. Overall, these findings expand our understanding of how autophagy supports cancer progression.

Although autophagy inhibition suppresses invasion in 3D culture, it does not ubiquitously revert oncogenic RAS-driven changes in cell behavior. Indeed, MAPK activation remains unaltered following autophagy inhibition in this 3D culture model, and, moreover, the oncogenic activation of RAS continues to disrupt fundamental aspects of 3D morphogenesis in autophagy-deficient cells. First, autophagy inhibition does not alter the ability of HRAS^{V12} to suppress apoptosis in 3D culture. Moreover, autophagy inhibition does not suppress proliferation in HRAS^{V12} 3D cultures; rather, the spherical structures from HRAS^{V12} ATG knockdown cells remain highly proliferative over extended periods. Remarkably, both others and we have shown that ATG depletion reduces soft-agar growth and attenuates the proliferation of RAS-transformed cells grown in monolayer (3–5, 30); hence, the absence of proliferative suppression in this 3D culture model may be context dependent. These results also differ from those obtained in *KRAS*-mutant mouse cancer models in which genetic *ATG* deletion impairs proliferation and, in certain cases, enhances apoptosis (4, 6, 7). Certain reasons may explain these differences. First, we have only reduced ATGs using RNA interference (RNAi), rather than genetically eliminated these proteins. Second, the experiments here are of significantly shorter duration in comparison with autophagy-deficient *KRAS*-mutant tumor growth *in vivo*.

Although previous studies have demonstrated that autophagy supports the invasion of glioblastoma cells, the mechanistic underpinnings remain unclear (31, 32). Cell

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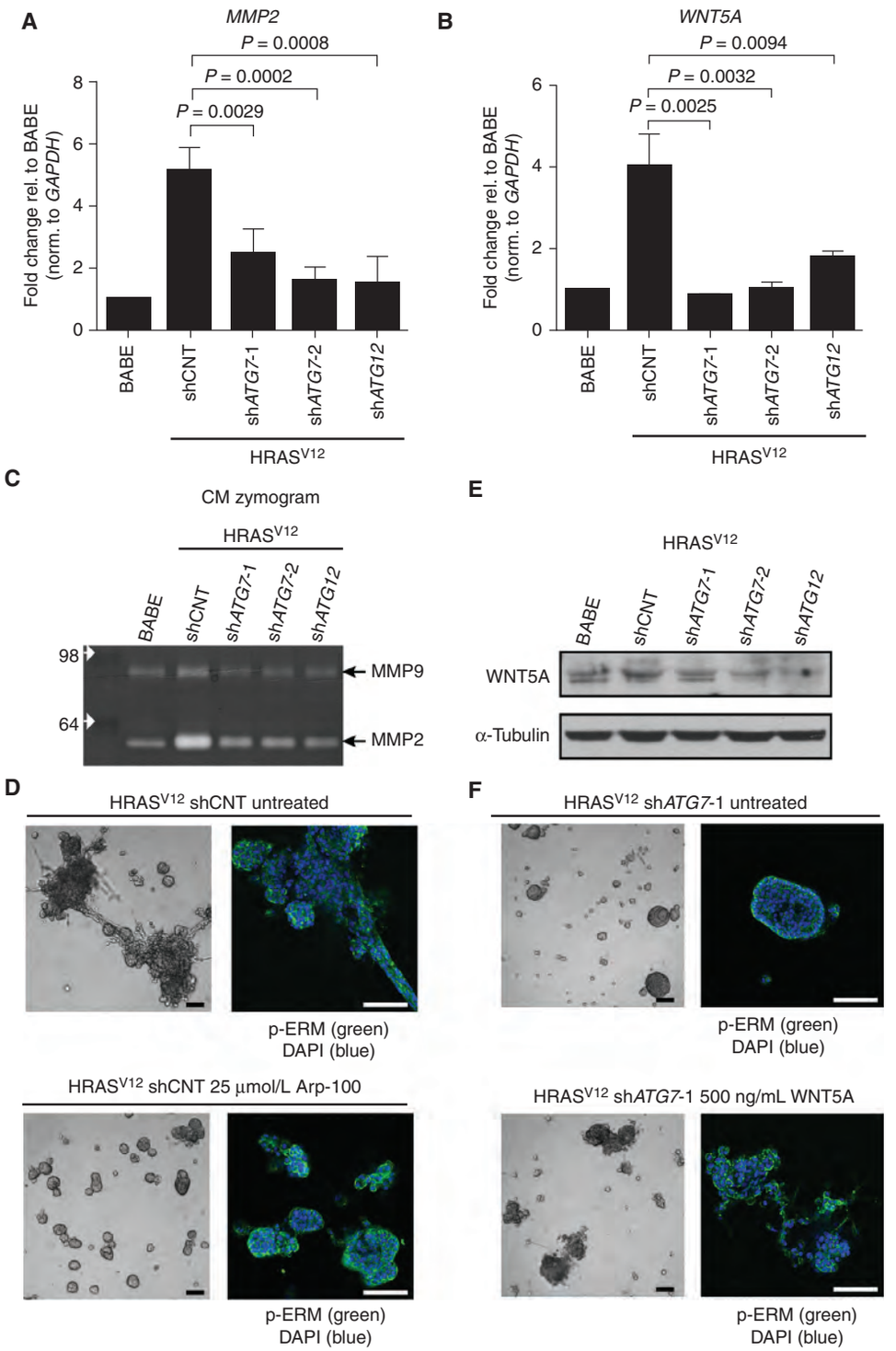

Figure 7. WNT5A and MMP2 are reduced following autophagy inhibition in 3D culture. **A** and **B,** RNA was isolated from BABE, HRAS^{V12} shCNT, and HRAS^{V12} sh*ATG* cells cultured in 3D for 8 days. Expression levels of *MMP2* and *WNT5A* were determined by qPCR and normalized to an internal control *GAPDH*. Results represent the mean relative to BABE ± SD (*MMP2*, $n = 4$; *WNT5A*, $n = 3$; Student *t* test). **C,** conditioned medium (CM) was collected from BABE, HRAS^{V12} shCNT, and HRAS^{V12} sh*ATG* cells grown in 3D culture. Activity levels of MMP9 and MMP2 in the conditioned media were determined by zymography. **D,** HRAS^{V12} shCNT cells were grown in the absence (top) or presence (bottom) of 25 μmol/L Arp-100. Left, structures were imaged on day 8 by phase contrast microscopy. Right, representative confocal images of structures immunostained with anti-phospho-Ezrin/Radixin/Moesin (p-ERM) to detect cell borders and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm. **E,** BABE, HRAS^{V12} shCNT, and HRAS^{V12} sh*ATG* cells were collected from 3D culture on day 8, lysed, and protein levels of WNT5A were determined by immunoblot analysis. **F,** HRAS^{V12} sh*ATG7*-1 cells were grown in 3D for 8 days in the absence (top) or presence (bottom) of 500 ng/mL WNT5A. Left, representative phase contrast images. Right, representative confocal images of structures immunostained with anti-phospho-ERM to detect cell borders and counterstained with DAPI. Scale bars, 100 μm.

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invasion requires the production and secretion of factors that stimulate migration and degrade the surrounding ECM (24). Upon treatment of autophagy-depleted HRAS^{V12} cells with conditioned media produced from their autophagy-competent counterparts, the ability to form invasive protrusions is completely restored, suggesting that autophagy is required for the efficient production of secreted factors that promote invasion and migration of HRAS^{V12} cells. Notably, conditioned media treatment does not promote invasion in nontransformed BABE cells, indicating that oncogenic RAS pathway activation is still required for invasion.

Importantly, we identify IL-6 as one critical factor whose secretion is ATG dependent; our results substantiate that this proinvasive cytokine is necessary to restore invasion in autophagy-deficient HRAS^{V12} cells. They also point to a specific role for autophagy in facilitating IL-6 secretion; upon ATG knockdown, RAS-transformed cells fail to secrete IL-6 into the conditioned media, yet both IL-6 transcription and translation remain intact. These results differ from recent studies of oncogenic RAS-mediated senescence, in which reduced IL-6 secretion in autophagy-deficient cells is proposed to be secondary to decreased protein synthesis (25, 29).

Although it has been traditionally viewed as an autodigestive process, growing evidence suggests new roles for autophagy in both conventional and unconventional secretion (33). Indeed, a genetic role for ATGs has been implicated in (i) unconventional secretion of proteins lacking N-terminal endoplasmic reticulum signal sequences (34–37), (ii) efficient egress of secretory lysosomes (38, 39), and (iii) conventional secretion of growth factors (40, 41). Further dissection of how autophagy directs the secretion of IL-6 and other factors during RAS transformation remains an important topic for future study. Remarkably, IL-6 re-addition only partially restores invasion and mesenchymal differentiation in HRAS^{V12} autophagy-deficient cultures, indicating that other factors promote invasion. In support of this possibility, these cells exhibit reduced levels of other proinvasive molecules, including WNT5A and MMP2. In contrast to reduced IL-6 secretion, which is likely a proximal event following ATG knockdown, these changes in WNT5A and MMP2 result from decreased gene expression, indicating that autophagy inhibition produces broader transcriptional changes contributing to reduced invasion by HRAS^{V12} cells.

Recently, the deletion of *RB1CC1/FIP200*, a gene mediating autophagosome initiation, was demonstrated to reduce lung metastases in the MMTV-PyMT breast cancer model. However, as *RB1CC1* deletion profoundly restricted primary tumor growth, it was unclear whether decreased metastasis was secondary to reduced primary tumor burden (12). In addition, although liver-specific deletion of *ATG7* or *ATG5* initiates the development of benign adenomas, these tumors are unable to progress to adenocarcinomas, suggesting that autophagy is required for advanced tumor progression (42, 43). Here, in epithelial cells transformed with oncogenic RAS, we demonstrate that defective autophagy results in decreased invasion and migration, which correlates with the reduced ability to metastasize *in vivo*. Although our results do not rule out potentially important functions for autophagy in disseminated cell survival or outgrowth at foreign tissue sites, they delineate new roles for autophagy in the control of secretion during carcinoma progression.

METHODS

Cell Lines

MCF10A cells were obtained from the American Type Culture Collection (ATCC) and cultured as previously described (44). MDA-MB-231 cells were obtained from the ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, penicillin, and streptomycin. Cell lines were passaged for less than 6 months following resuscitation and were not authenticated.

3D Culture Assays

MCF10A overlay 3D culture was performed as previously described (44). As indicated, the following reagents were added to cultures: 500 ng/mL WNT5A (R&D Systems), 200 ng/mL IL-6 (PeproTech), 25 μg/mL anti–IL-6 function–blocking antibody (R&D Systems), 25 μg/mL IgG control antibody (BD Biosciences), 25 μmol/L Arp-100 (Santa Cruz Biotechnology), 5 μmol/L chloroquine diphosphate salt (Sigma), and 5 nmol/L bafilomycin A (Sigma). For the 3D ECM degradation assay, human DQ-COL4 (Invitrogen) was mixed with Matrigel to a final concentration of 25 μg/mL before plating. To collect cells for immunoblotting and RNA isolation, cultures were incubated with 0.25% Trypsin/EDTA at 37°C for 10 minutes to dissociate cells from surrounding matrix and create a single-cell suspension. Cells were resuspended in media containing 20% serum and washed twice with PBS to remove residual Matrigel.

For coculture assays, sh*ATG7*-1 was expressed in HRAS^{V12} cells stably expressing pBABEhygro–GFP. This GFP-labeled "target" cell line was then cultured in isolation or combined with unlabeled (pBABEhygro) HRAS^{V12} shCNT cells at a ratio of 3:1, with total cell number kept constant at 7,500 cells per well. For conditioned media experiments, HRAS^{V12} sh*ATG*-expressing cells were grown in 3D culture for 3 days; subsequently, the media was replaced with conditioned media harvested from BABE or HRAS^{V12} shCNT MCF10A cells grown in 3D culture for 6 to 8 days. When indicated, 25 μg/mL anti–IL-6 function–blocking or IgG isotype control antibody was added to the conditioned media.

Wound-Healing Assay

Cells were grown to confluence in 3.5-cm dishes and incubated overnight in assay media lacking EGF for MCF10A cells or DMEM + 2% FBS for MDA-MB-231 cells. Wound healing was performed in the presence of 2 μg/mL mitomycin C (Sigma). Cells were wounded with a 200-μL pipette tip and imaged at the time of wounding (0 hours) and the indicated time points. Average wound widths were measured at each time point, and decreases in wound width were calculated by subtracting the average width at the final time point from the average width at 0 hours using MetaMorph Software (v6.0).

Transwell Assay

Cells were starved overnight in assay media lacking EGF and then plated at 1.0×10^5 in the top chamber of an 8-μm Transwell filter in assay media lacking EGF. The bottom chamber was filled with assay medium containing 5 ng/mL EGF. Cells were allowed to migrate for 24 hours, after which the top of each filter was cleared of cells. Cells attached to the bottom of the filter were fixed and stained with crystal violet. Crystal violet was extracted with 10% acetic acid and the absorbance was measured at 600 nm.

Experimental Metastasis Assay

For experimental metastasis assays, cells were infected with pHIV-ZsGreen (Addgene; plasmid 18121). A total of 1.0×10^6 HRAS^{V12} shCNT, sh*ATG7*-1, and sh*ATG12* cells stably expressing ZsGreen were injected into the tail vein of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. After 140 days, whole lungs were fixed and imaged to detect the number of ZsGreen-positive foci per lung. All animal experiments were conducted in accordance with approved

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protocols of the University of California, San Francisco (UCSF; San Francisco, CA) Institutional Animal Care and Use Committee (IACUC).

IL-6 ELISA

Day 5 3D cultures were washed twice with PBS and cultured for 18 hours in serum-free media. Conditioned medium was collected, and total protein levels were determined by BCA assay (Thermo Scientific) to normalize samples. IL-6 levels were measured using the Quantikine High Sensitivity ELISA Kit (R&D Systems).

Statistical Analyses

Each experiment was repeated at least three independent times. GraphPad Prism software (v5.0b) was used for generation of graphs and statistical analyses. *P* values were determined by a Student *t* test or ANOVA as stated.

Disclosure of Potential Conflicts of Interest

J. Debnath has received honoraria from the Speakers' Bureaus of Amgen and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R. Lock, C.M. Kenific, J. Debnath
Development of methodology: R. Lock, C.M. Kenific, J. Debnath
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Lock, C.M. Kenific, A.M. Leidal, E. Salas, J. Debnath
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Lock, C.M. Kenific, A.M. Leidal, E. Salas, J. Debnath
Writing, review, and/or revision of the manuscript: R. Lock, A.M. Leidal, J. Debnath
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Lock
Study supervision: J. Debnath

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Autophagy dependent production of secreted factors facilitates oncogenic Ras-driven invasion

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Retroviral vectors and retrovirus production: pBABEneo $HRAS^{V12}$ has been previously described (1). pBABEhygro GFP was generated from pBABEpuro GFP. For retroviral transduction, VSV-G-pseudotyped retroviruses were generated, and cells were infected and selected as previously described (2).

shRNA and siRNA. pLKO.1 lentiviral expression plasmids containing short hairpin RNAs against ATG7 and ATG12 were purchased from Sigma (Mission shRNA). The production of viral particles and the generation of cell lines expressing shATGs have been described (1). The target sequences for hairpins directed against human ATG7 (NM_006395) are: shATG7-1 (TRCN0000007584): GCCTGCTGAGGAGCTCTCCA; and shATG7-2 (TRCN0000007587): CCCAGCTATTGGAACACTGTA; directed against human ATG12 (NM_004707) is: shATG12 (TRCN0000007394): TGGAACTCTCTATGAGTGTTT; directed against human ATG3 (NM_022488) is: shATG3 (TRCN0000149597): CCTACCAACAGGCAAACAATT. For siRNA-mediated knockdown of autophagy genes in MDA-MB-231 cells, siGenome SMARTpool siRNAs against human *ATG7* (M-020112-01) and human *ATG12* (M-010212-02) were purchased from Dharmacon and cells were transfected as previously described (3).

Phase and immunofluorescence image acquisition and analysis. 3D cultures were fixed and stained as previously described (2). Phase and indirect immunofluorescent imaging were performed on an Axiovert 200 microscope (Carl Zeiss) with 4x (NA: 0.1) or 10x (NA:0.25) lenses, Spot RT camera (Diagnostic Instruments) and mercury lamp, and images were acquired using MetaMorph software (v6.0). Confocal analyses were performed using a C1Si confocal laser-scanning microscope (Nikon) with 20x (NA:0.75) or 60x (NA:1.2) lenses and images were collected using EZ-C1 software (v3.20). The following antibodies and reagents were used for staining: anti-Ki67 (Invitrogen), anti-cleaved caspase 3 (CASP3, Cell Signaling), anti-β–catenin (CTNNB1, BD Biosciences), anti-laminin 5, human specific (LAMA5, EMD Millipore), anti-phospho-ERM (Cell Signaling), phalloidin-488 and phalloidin-546 (Invitrogen), DAPI (Sigma), Alexa Fluor goat anti-mouse-488 and 568 and Alexa Fluor goat anti-rabbit-488 and 568 (Invitrogen). Quantification of invasive protrusions was performed according to a method described in (4). Briefly, 3D cultures were analyzed at 48h treatment with conditioned media or recombinant human IL6; at this time point, invasive protrusions emanating from individual globular structures were easily discernable, but complex intermingled networks between structures had not formed yet. At least 200 structures in each 3D culture assay were analyzed and the percent of structures with evidence of least one invasive protrusion was quantified; for each condition, at least 3 independent experiments were analyzed.

Immunoblotting. Cells were lysed in RIPA buffer plus 10mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_3, 10 nM calyculin A, and protease inhibitors. Lysates were clarified by centrifugation for 15 min at 4°C, and protein concentrations were assessed using a BCA protein

assay (Thermo). Samples containing equal amounts of protein were boiled in SDS sample buffer (15-50μg of total protein per lane), resolved using SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride membrane. Membranes were blocked in PBS + 0.1% Tween 20 with 5% nonfat dry milk, incubated with the primary antibodies indicated overnight at 4°C, washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and analyzed by enhanced chemiluminescence. The following primary antibodies were used: anti-RAS (BD Biosciences), anti-phospho-ERK1/2 (MAPK) (Invitrogen), anti-total-ERK1/2 (MAPK, Invitrogen), anti-ATG7 (Santa Cruz Biotechnology), anti-ATG5 (Cell Signaling), anti-ATG3 (Sigma), anti-LC3 (Sigma), anti-IL6 (Abcam), anti-Keratin 14 (KRT14, Novus Biologicals), anti-vimentin (VIM, BD Biosciences), anti-E-cadherin (CDH1, BD Biosciences), anti-Fibronectin (FN1, Sigma), anti-N-cadherin (CDH2, BD Biosciences), anti-WNT5A (R&D Systems), anti-p62 (Progen Biotechnik), anti-α-tubulin (TUBA, Sigma), anti-β-actin (ACTB, Sigma), and anti-GAPDH (Santa Cruz Biotechnology). In certain experiments, a previously described anti-LC3 rabbit polyclonal antibody was used (3) which is now commercial available (EMD Millipore).

qPCR array and qPCR. Cells were collected from 3D on day 8 and RNA was isolated using Qiagen RNeasy kit. 50ng of total RNA was used for each reaction and cDNA synthesis and PCR amplification were performed using the Brilliant II SYBR Green qRT-PCR Master Mix Kit (Stratagene). qPCR reactions were run using a StepOnePlus thermal cycler (Applied Biosciences) and analyzed using StepOne software (v2.2). For each set of experiments samples were run in triplicate, expression levels were determined based on a standard curve run with each primer set, and levels were normalized to an internal control, GAPDH. The following are the

primer sequences used: *GAPDH*; For: 5'-CATGTTCGTCATGGGTGTGAACCA-3' Rev: 5'ATGGCATGGACTGTGGTCATGAGT-3', *MMP2*; For: 5'-AGAAGGATGGCAAGTACGGCTTCT-3' Rev: 5'-AGTGGTGCAGCTGTCATAGGATGT-3', *WNT5A*; For: 5'-CGCCCAGGTTGTAATTGAAGCCAA-3' Rev: 5'-TGTCCTTGAGAAAGTCCTGCCAGT-3', *IL6*; For: 5'-TGAAAGCAGCAAAGAGGCACT-3' Rev: 5'-TGAATCCAGATTGGAAGCATCC-3'.

Conditioned media zymography. On day 7, 3D structures were washed twice with PBS and cultures were incubated with serum free media overnight. Conditioned media was collected, the total protein level was determined by BCA assay (Thermo Scientific), and samples were normalized accordingly. Gelatin zymography using the prepared conditioned media samples was performed as previously described (5).

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. **ATG knockdown inhibits Ras-driven invasion in 3D culture.** (A) Representative phase contrast images of MCF10A cells expressing an empty vector control (BABE) grown in 3D culture for the indicated times. Bar, 100μm. (B) Protein levels of ATG7, ATG5 (ATG5:12 complex), and ATG3 in $HRAS^{V12}$ MCF10A cells expressing shATGs. (C) Protein levels of LC3-I and LC3-II in $HRAS^{V12}$ cells expressing shATGs. (D) Levels of p62 in $HRAS^{V12}$ cells expressing shATGs collected from 3D culture on day 8. (E) Levels of LC3-I and LC3-II in $HRAS^{V12}$ shCNT or shATG expressing cells grown in full media or starved in HBSS in the presence or absence of E64d and pepstatin A (E/P). (F) Representative 3D culture phase contrast images of $HRAS^{V12}$ cells expressing shATG3 or grown in the presence of 5μM chloroquine or 5nM bafilomycin A (BafA). Bar, 100μm. (G) Levels of RAS and phosphorylated ERK1/2 in BABE, $HRAS^{V12}$ shCNT, and $HRAS^{V12}$ shATG cells collected from 3D culture on day 8. (H) Protein levels of ATG7, ATG5 (ATG5:12 complex), LC3-I and LC3-II in MDA-MB-231 cells following siRNA knockdown of ATG7 and ATG12.

Figure S2. **Effects of ATG depletion on mesenchymal differentiation and junctional integrity in $HRAS^{V12}$ MCF10A cells.** (A) Protein levels of keratin 14 (KRT14), vimentin (VIM), E-cadherin (CDH1), fibronectin (FN1) and N-cadherin (CDH2) in BABE, $HRAS^{V12}$ shCNT or $HRAS^{V12}$ shATG cells collected from 3D cultures on day 8. (B) Two representative confocal images of day 8 BABE, $HRAS^{V12}$ shCNT or $HRAS^{V12}$ shATG 3D cultures immunostained with anti-β-catenin (CTNNB1) and counterstained with DAPI. Note that only isolated focal areas of junctional β-catenin staining are detected in $HRAS^{V12}$ shCNT cultures,

whereas robust β-catenin staining is evident at cell-cell junctions throughout BABE and $HRAS^{V12}$ shATG cultures. Bar, 50μm.

Figure S3. **Effects of conditioned media treatment and recombinant human IL6 re-addition on BABE and $HRAS^{V12}$ shATG 3D cultures.** (A) Quantification of invasive protrusions in $HRAS^{V12}$ shATG cells following treatment with the indicated conditioned media (CM) for 48h; the percent of structures in a culture with one or more invasive protrusion was enumerated. **P<0.01, ***P<0.001 (Student's t test, n=3). (B) Nontransformed MCF10A cells expressing empty vector (BABE) were cultured in 3D for 3d and subsequently treated with BABE or $HRAS^{V12}$ shCNT conditioned media (CM). Representative phase contrast images at 24h and 72h following the addition of CM. Bar, 100μm. (C) Representative phase contrast images of BABE 3D cultures grown in the presence or absence of 200ng/mL recombinant human IL6 (rhIL6). Bar, 100μm. (D) 3D cultures of $HRAS^{V12}$ shATG cells were treated with 200ng/ml rhIL6, fixed and immunostained with an antibody against LAMA5 and DAPI counterstained. Bar, 50μm. (E) Quantification of invasive protrusions in $HRAS^{V12}$ shATG cells following treatment with 200ng/ml rhIL6; the percent of structures in a culture with one or more invasive protrusion was enumerated. *P<0.05, ***P<0.001 (Student's t test, n=3). (Student's t test, n=3). (F) Effects of rhIL6 treatment on keratin 14 (KRT14) and vimentin (VIM) protein levels in $HRAS^{V12}$ shCNT and $HRAS^{V12}$ shATG7-1 cells grown in 3D culture in the presence or absence of 200ng/mL recombinant human IL6 (rhIL6). (G) Fold change in *MMP2* and *WNT5A* expression in cells isolated from $HRAS^{V12}$ shATG7-1 3D cultures following treatment with 200ng/ml rhIL6. Results represent the fold change relative to untreated cultures (mean +/- s.d.). ns, nonsignificant. (*MMP2,* n=4; *WNT5A*, n=4, Student's t-test).

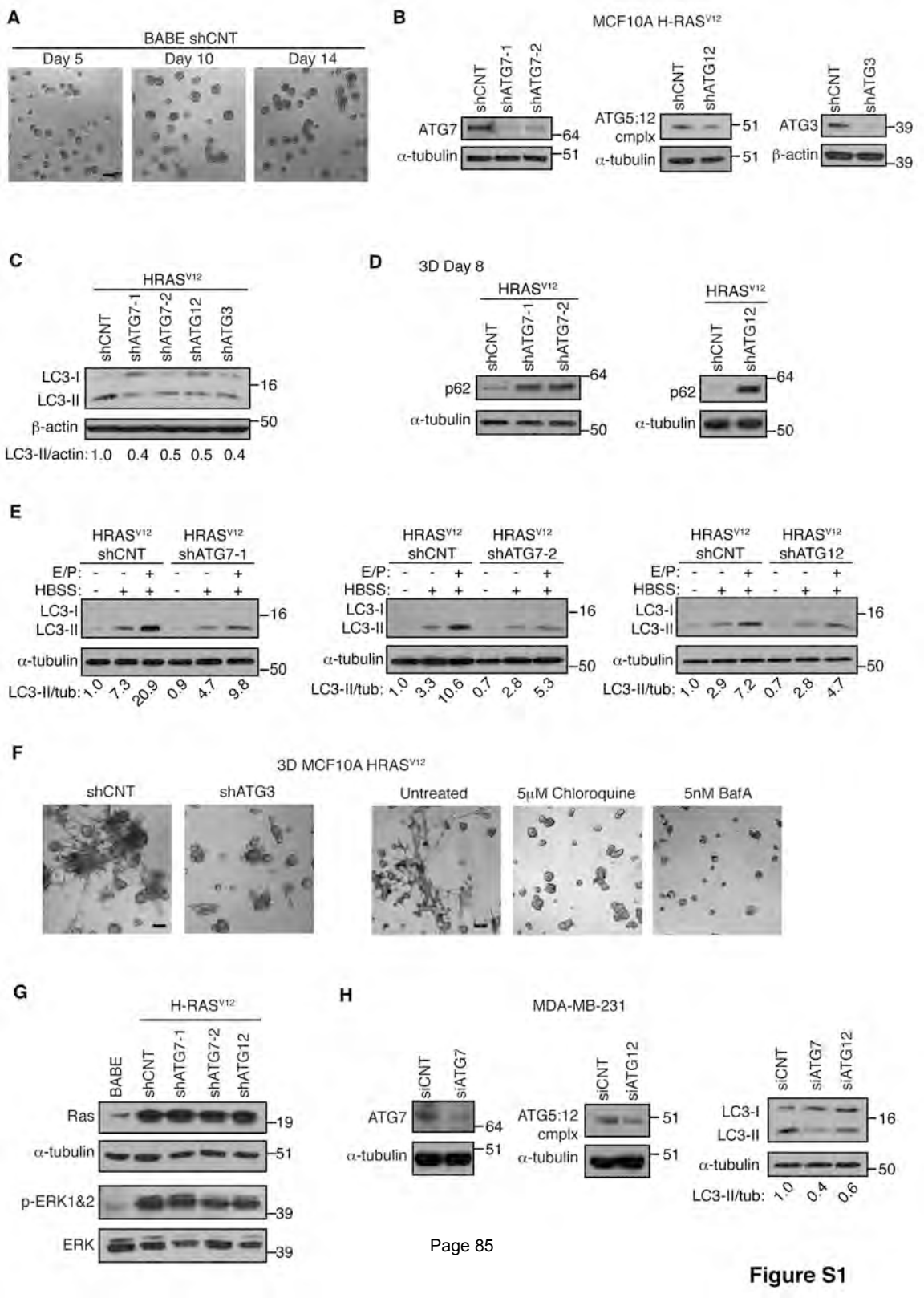
A
BABE shCNT
Day 5
Day 10
Day 14
B
MCF10A H-RASV12
shCNT
shATG7-1
shATG7-2
ATG7
64
α-tubulin
51
shCNT
shATG12
ATG5:12
cmplx
51
α-tubulin
51
shCNT
shATG3
ATG3
39
β-actin
39
C
HRASV12
shCNT
shATG7-1
shATG7-2
shATG12
shATG3
LC3-I
LC3-II
16
β-actin
50
LC3-II/actin: 1.0 0.4 0.5 0.5 0.4
D
3D Day 8
HRASV12
shCNT
shATG7-1
shATG7-2
p62
64
α-tubulin
50
HRASV12
shCNT
shATG12
p62
64
α-tubulin
50
E
HRASV12
shCNT
HRASV12
shATG7-1
E/P:
HBSS:
LC3-I
LC3-II
16
α-tubulin
50
LC3-II/tub: 1.0 7.3 20.9 0.9 4.7 9.8
HRASV12
shCNT
HRASV12
shATG7-2
E/P:
HBSS:
LC3-I
LC3-II
16
α-tubulin
50
LC3-II/tub: 1.0 3.3 10.6 0.7 2.8 5.3
HRASV12
shCNT
HRASV12
shATG12
E/P:
HBSS:
LC3-I
LC3-II
16
α-tubulin
50
LC3-II/tub: 1.0 2.9 7.2 0.7 2.8 4.7
F
3D MCF10A HRASV12
shCNT
shATG3
Untreated
5μM Chloroquine
5nM BafA
G
H-RASV12
BABE
shCNT
shATG7-1
shATG7-2
shATG12
Ras
19
α-tubulin
51
p-ERK1&2
39
ERK
39
H
MDA-MB-231
siCNT
siATG7
ATG7
64
α-tubulin
51
siCNT
siATG12
ATG5:12
cmplx
51
α-tubulin
51
siCNT
siATG7
siATG12
LC3-I
LC3-II
16
α-tubulin
50
LC3-II/tub: 1.0 0.4 0.6

Figure S1

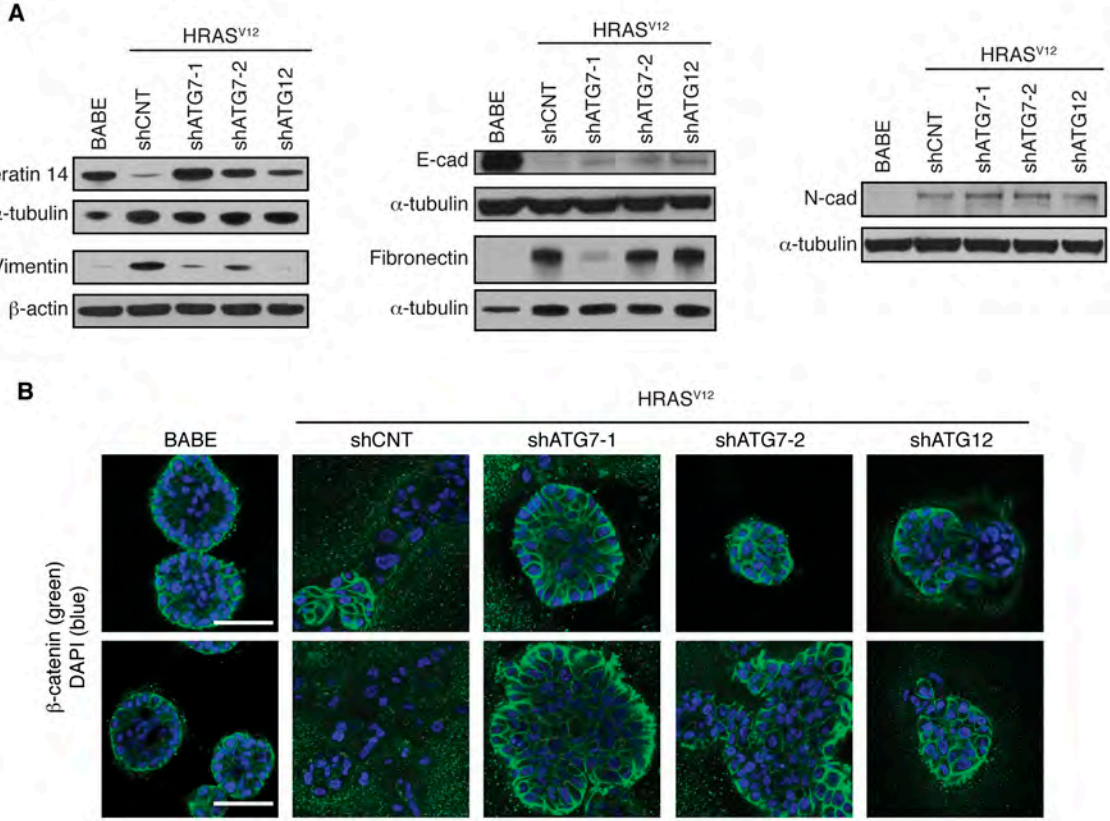
A
HRASV12
BABE
shCNT
shATG7-1
shATG7-2
shATG12
Keratin 14
α-tubulin
Vimentin
β-actin
E-cad
α-tubulin
Fibronectin
α-tubulin
N-cad
α-tubulin
B
HRASV12
BABE
shCNT
shATG7-1
shATG7-2
shATG12
β-catenin (green)
DAPI (blue)

Figure S2

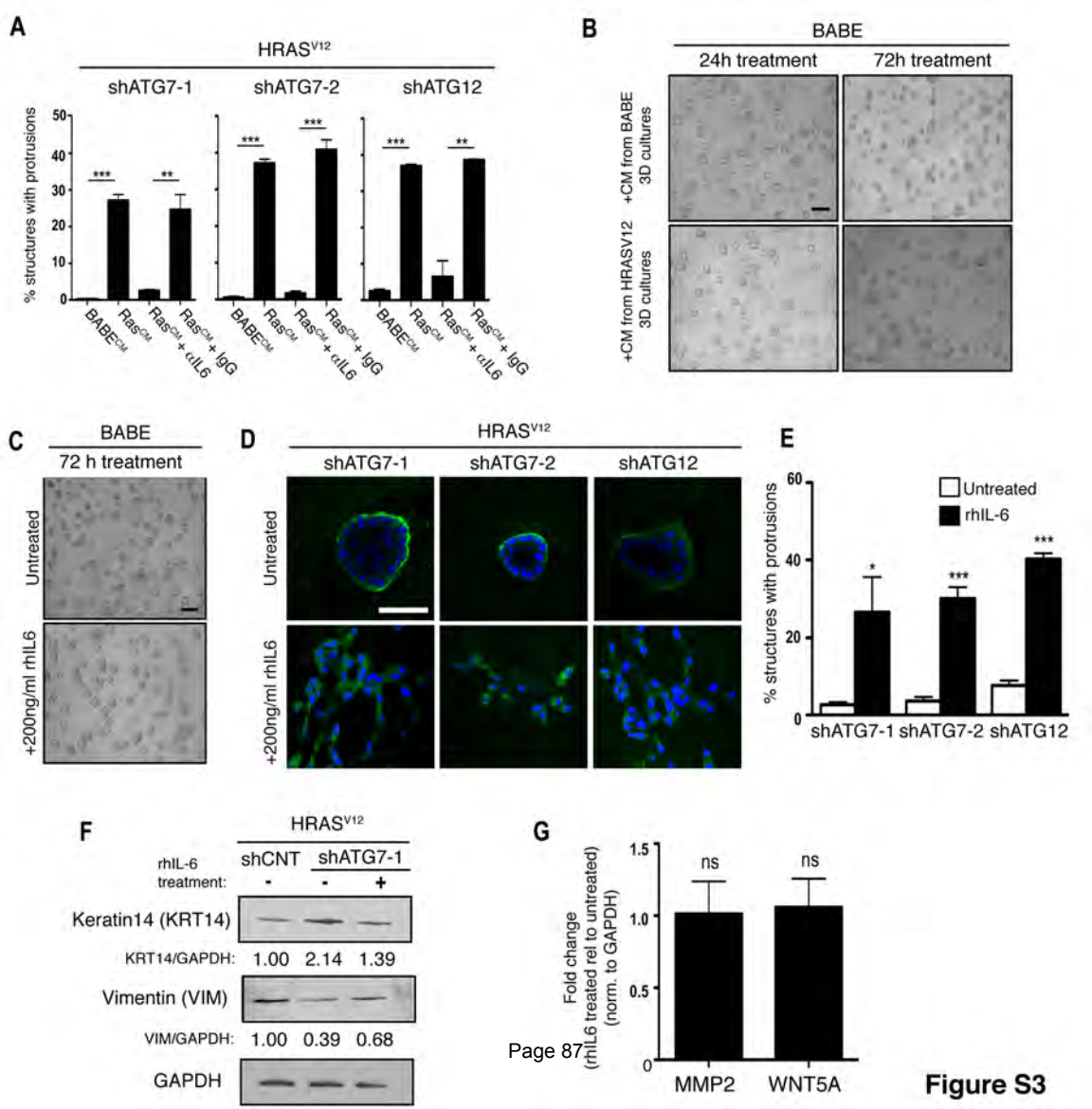
A
HRASV12
shATG7-1
shATG7-2
shATG12
% structures with protrusions
BABECM
RasCM
RasCM + IgG
RasCM + αIL6
B
BABE
24h treatment
72h treatment
+CM from BABE 3D cultures
+CM from HRASV12 3D cultures
C
BABE
72 h treatment
Untreated
+200ng/ml rhIL6
D
HRASV12
shATG7-1
shATG7-2
shATG12
Untreated
+200ng/ml rhIL6
E
Untreated
rhIL-6
% structures with protrusions
shATG7-1
shATG7-2
shATG12
F
HRASV12
rhIL-6 treatment:
shCNT
shATG7-1
Keratin14 (KRT14)
KRT14/GAPDH: 1.00 2.14 1.39
Vimentin (VIM)
VIM/GAPDH: 1.00 0.39 0.68
GAPDH
G
Fold change (rhIL6 treated rel to untreated) (norm. to GAPDH)
ns
MMP2
WNT5A

Figure S3

CHAPTER TWO

Autophagy and Cancer Metabolism

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Abstract

The metabolism of malignant cells is profoundly altered in order to maintain their survival and proliferation in adverse microenvironmental conditions. Autophagy is an intracellular recycling process that maintains basal levels of metabolites and biosynthetic intermediates under starvation or other forms of stress, hence serving as an important mechanism for metabolic adaptation in cancer cells. Although it is widely acknowledged that autophagy sustains metabolism in neoplastic cells under duress, many questions remain with regard to the mutual relationship between autophagy and metabolism in cancer. Importantly, autophagy has often been described as a "double-edged sword" that can either impede or promote cancer initiation and progression. Here, we overview such a dual function of autophagy in tumorigenesis and our current understanding of the coordinated regulation of autophagy and cancer cell metabolism in the control of tumor growth, progression, and resistance to therapy.

1. INTRODUCTION

The ability of cells to adapt to stress requires diverse changes in cellular metabolism. One of the principal pathways contributing to this metabolic adaptive response is macroautophagy (commonly termed autophagy), a tightly regulated lysosomal digestion process. Because degradation through autophagy allows recycling of nutrients, autophagy serves as an important survival and fitness pathway induced by a wide array of stresses including nutrient deprivation, growth factor withdrawal, oxidative stress, infection, and hypoxia (Avivar-Valderas et al., 2011; Boya, Reggiori, & Codogno, 2013; Lum et al., 2005; Yin, Kharbanda, & Kufe, 2009). In addition to its role in the stress-induced response, autophagy plays an essential homeostatic function by selectively removing damaged or nonfunctional proteins and organelles. These quality control functions have been demonstrated to be especially crucial in certain cell types: Liver cells are reliant on autophagy for the breakdown of stored metabolites, pancreatic β-cells utilize autophagy to manage high levels of endoplasmic reticulum (ER) stress, and postmitotic neurons require autophagy to remove potentially damaging proteins that cannot be diluted by cell division (Hara et al., 2006; Jung et al., 2008; Komatsu et al., 2005, 2006; Wu et al., 2009).

Given these key functions of autophagy in normal cells and tissues, it is not surprising that disruptions in autophagy have been implicated in numerous human diseases, including neurodegeneration, liver disease, inflammation,

type 2 diabetes, and cancer (Debnath, 2011; Levine & Kroemer, 2008; Murrow & Debnath, 2013). While autophagy has been demonstrated to improve disease outcome in many cases by facilitating stress-induced metabolic adaptation or cellular homeostasis, the role of autophagy is more complex in cancer. Autophagy serves as an important tumor suppressor mechanism that impedes cancer initiation; at the same time, autophagy can promote the survival of tumor cells in response to diverse microenvironmental and therapeutic stresses and support anabolic capacity in fast-replicating, metabolically stressed tumor cells (Kimmelman, 2011; Levine & Kroemer, 2008; Rabinowitz & White, 2010). Despite this widely accepted notion that autophagy critically fuels metabolism in tumor cells under duress, many questions remain with regard to the interrelationships between autophagy and metabolism in cancer. This chapter focuses on the coordinated regulation of autophagy and cancer cell metabolism, controlling tumor growth, progression, and resistance.

2. OVERVIEW OF THE AUTOPHAGY MACHINERY

The process of macroautophagy occurs in a series of distinct steps: (1) initiation of the isolation membrane; (2) nucleation; (3) elongation of the double-membrane structure to form the autophagosome; and (4) fusion to the lysosome to form an autolysosome, in which the contents are degraded (Fig. 2.1). Studies in yeast have revealed over 30 autophagy related genes and proteins (ATGs and Atgs respectively) involved in the autophagic trafficking process, many of whose mammalian orthologues have also been identified (Nakatogawa, Suzuki, Kamada, & Ohsumi, 2009). This section provides an overview of the key molecular complexes that comprise the autophagy machinery in mammalian cells—more detailed reviews can be found elsewhere (Klionsky, 2013; Klionsky & Emr, 2000; Yang & Klionsky, 2010).

2.1. Initiation and the ULK complex

In mammals, autophagosome initiation requires the ULK complex, which consists of ULK1/2 (orthologous to yeast Atg1) associated with ATG13, FIP200, and ATG101 (Mizushima, 2010; Fig. 2.1A). At least three different ULK proteins are involved in different aspects of autophagy, among which ULK1/2 bear the highest similarity to yeast Atg1. Under nutrient-rich conditions, the ULK complex interacts with mTORC1 and remains inactivated

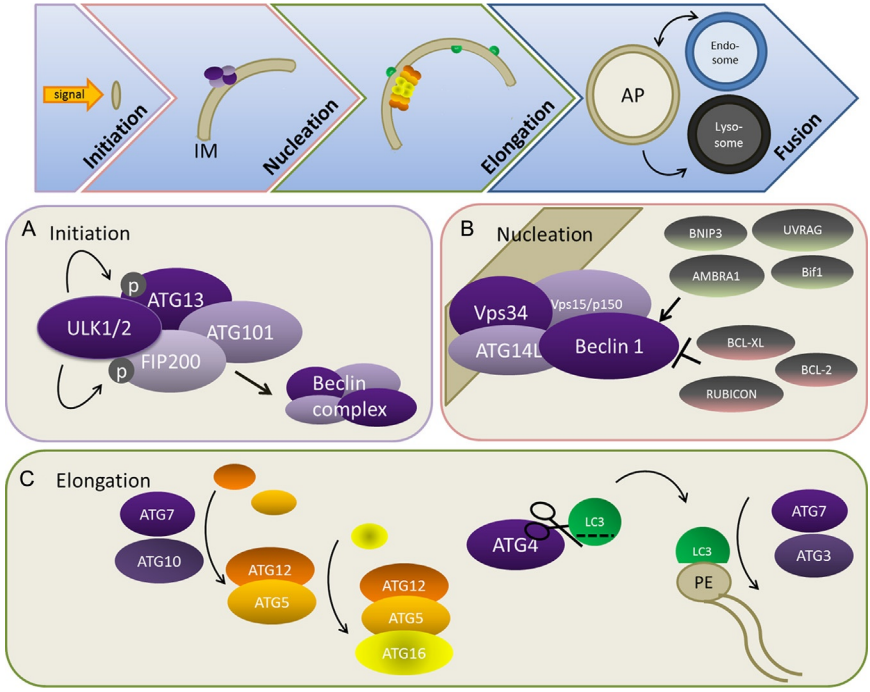

Figure 2.1 *Overview of the autophagy trafficking process*. The process of macroautophagy occurs in a series of distinct steps: (1) initiation of the isolation membrane (IM); (2) nucleation; (3) elongation of the double-membrane structure to form the autophagosome (AP); and (4) fusion to endosomes and lysosomes ultimately results in the formation of an autolysosome, in which the contents are degraded. (A) Initiation is mediated by the ULK complex. Activation of ULK activity leads to the phosphorylation of FIP200 and ATG13 and initiates nucleation via interaction with the Beclin 1 complex. (B) Beclin 1/VPS34/ATG14/PIK3R4 (p150) complex interacts with multiple interacting partners that positively and negatively regulate Beclin 1/VPS34 lipid kinase activity, resulting in the fine-tuning of autophagosome nucleation. (C) Elongation requires two ubiquitin-like conjugation pathways that form the ATG12–ATG5/ATG16 complex and phosphatidylethanolamine (PE)-conjugated LC3.

by mTORC1-mediated phosphorylation. However, upon nutrient deprivation, mTORC1 dissociates from the complex resulting in the dephosphorylation of inhibitory sites and concomitant autophosphorylation of activating sites in ULK1/2 (Chan, 2009). The kinase activation of ULK1/2 then leads to the phosphorylation and activation of ATG13 and FIP200 (Jung et al., 2009). The active complex then initiates nucleation by interaction with the Beclin 1/ATG14/VPS34 complex.

2.2. Nucleation and Beclin 1/ATG14/VPS34 complex

The formation of autophagosomes requires the activity of class III phosphatidylinositol 3-kinase (PI3K) VPS34, which is essential for phosphatidylinositol 3-phosphate production during the early stages of phagophore nucleation. VPS34 forms a complex with the yeast Atg6 orthologue Beclin 1, ATG14L, and VPS15/PIK3R4 (p150) (Itakura, Kishi, Inoue, & Mizushima, 2008; Zhong et al., 2009). Various binding partners of Beclin 1 have been identified (Fig. 2.1B), including UV irradiation resistance-associated gene (UVRAG) (Itakura et al., 2008; Liang et al., 2006), ATG14L/Barkor (Matsunaga et al., 2009; Zhong et al., 2009), and AMBRA1 (Fimia et al., 2007), all of which positively regulate Beclin 1 activity. Notably, ATG14L plays a critical role in specifying the site of the VPS34 complex relocation and therefore phagophore nucleation (Matsunaga et al., 2009). UVRAG also interacts with SH3GLB1/Bif-1 (an N-BAR domain protein), which potentially leads to phagophore membrane curvature, and expedites autophagosome–lysosome fusion (Liang et al., 2008; Takahashi et al., 2007). In addition to these positive regulators, other Beclin 1-interacting partners, including BCL-2, BCL-xL, Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein), AKT, and EGFR, are negative regulators of the Beclin 1/VPS34 autophagy-promoting complex (Matsunaga et al., 2009; Pattingre et al., 2005; Wang et al., 2012; Wei et al., 2013; Zhong et al., 2009). Overall, these studies indicate that multiple class III PI3K complexes exist concurrently within the cell, suggesting that these proteins can exquisitely tune the level of autophagy. Notably, several proteins in this complex have tumor-suppressive or antiproliferative effects, which are discussed in detail in the succeeding text.

2.3. Elongation and the ATG12/ATG8 conjugation systems

The elongation of the phagophore membrane requires two ubiquitin-like conjugation systems. In the first, ATG7 and ATG10 (E1- and E2-like enzymes, respectively) conjugate ATG12 to ATG5. The ATG5–ATG12 complex binds ATG16 and forms a large multimeric complex called the ATG16L complex, which is essential for the elongation of the nascent phagophore (Fig. 2.1C). The second conjugation system involves cleavage of the ubiquitin-like molecule, ATG8, by the protease ATG4 to expose a C-terminal glycine residue required for subsequent activation and conjugation reactions. Several mammalian orthologues of yeast Atg8 have been

identified, of which the best characterized is microtubule-associated protein 1 light chain 3 (LC3) alpha (MAP1LC3A) (Weidberg, Shpilka, Shvets, Shinder, & Elazar, 2010). Atg4 also has four mammalian isoforms, although the specificities are entirely known (Li et al., 2011; Shpilka, Weidberg, Pietrokovski, & Elazar, 2011). Ultimately, LC3 is conjugated to the lipid phosphatidylethanolamine (PE) via ATG7 and E2-like ATG3 and is subsequently recruited to both the outer and inner surfaces of the autophagosomal membrane (Fig. 2.1C). LC3 and other Atg8 family members can mediate membrane tethering and hemifusion, which may be important in the fusion of the ends of the phagophore membrane into a closed autophagosome (Weidberg et al., 2011).

In addition, LC3 is an important mediator for selectively targeting cargo for autophagic degradation. Several ubiquitin-binding proteins have been identified as cargo receptors for autophagy substrates (Johansen & Lamark, 2011), including p62/SQSTM1 (Bjørkøy et al., 2005), NBR1 (Kirkin et al., 2009), NDP52 (Thurston, Ryzhakov, Bloor, von Muhlinen, & Randow, 2009), and OPTN (Wild et al., 2011). These cargo receptors contain a well-conserved linear amino acid motif called the LIR (LC3-interacting region) that is necessary for specific targeting to the autophagosome. Interestingly, the LIR consensus sequence has been identified in a number of proteins, suggesting that the repertoire of LC3-interacting proteins acting as cargo receptors for selective autophagy may be expansive. In support of this, a large-scale proteomic study demonstrated that the mammalian ATG8 family has 67 high-confidence interactions with other cellular proteins (Berhends, Sowa, Gygi, & Harper, 2010).

2.4. Fusion

After an autophagosome forms, it fuses with the endosome or lysosome where the engulfed components may be recycled (Fig. 2.1E). Autophagosomes travel along microtubules, pushed by dynein, to lysosomes. Fusion requires ESCRT, SNAREs—specifically syntaxin 17 (Itakura, Kishi-Itakura, & Mizushima, 2012)—VPS family proteins, and RAB7. Fusion to the lysosome is the last step in the degradation of the intracompartmental components, and impaired lysosome function prevents complete autophagic flux. Hence, lysosomotrophic agents such as hydroxychloroquine (HCQ) are used experimentally to inhibit autophagy. These lysosomal inhibitors are proposed to impair autophagosome maturation and flux by altering the pH of the lysosome; nonetheless, it is important to recognize that these compounds impact a broad array of processes other than autophagy.

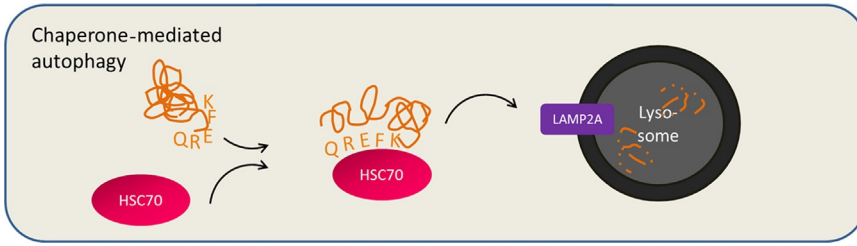

Figure 2.2 *Chaperone-mediated autophagy*. Chaperone-mediated autophagy is an additional route by which proteins are degraded in the lysosome. HSC70 binds to proteins with a KFERQ pentapeptide motif, assists in protein unfolding, and delivers the targeted protein directly to the lysosome for degradation via interaction with lysosome-associated membrane protein 2A (LAMP2A).

2.5. Chaperone-mediated autophagy

Although this chapter principally focuses on macroautophagy, it is important to recognize that multiple routes of autophagic degradation exist, including microautophagy and chaperone-mediated autophagy (CMA) (Mizushima, 2007). CMA warrants special attention because of its emerging role in cancer (Kon et al., 2011; Lv et al., 2011; Vakifahmetoglu-Norberg et al., 2013). CMA is a highly selective form of autophagy in which specific proteins are targeted to the lysosome via their interaction with a cytosolic chaperone protein—HSC70—that recognizes and binds to a specific pentapeptide motif, the KFERQ sequence. This interaction leads to binding to the lysosome via a variant of the lysosome-associated membrane protein 2A (LAMP2A), and after some unfolding, the targeted protein is directly delivered into the lysosome for degradation (Fig. 2.2; Cuervo, Terleckyh, Dice, & Knecht, 1994; Dice, Chiang, Spencer, & Backer, 1986; Koga, Martinez-vicente, Macian, Verkhusha, & Cuervo, 2011). Interestingly, CMA can be induced in mammalian cells when macroautophagy is inhibited and vice versa, indicating that a switch in one type of autophagy can compensate for a deficiency in the other (Massey, Kaushik, Sovak, Kiffin, & Cuervo, 2006; Wang et al., 2008).

3. METABOLIC STIMULI REGULATING AUTOPHAGY

Metabolic stresses often occur in solid tumors and the tumor microenvironment—rapidly multiplying tumor cells and tumors that have yet to initiate angiogenic programs and often cannot maintain nutrient supply and quickly become hypoxic. To forestall senescence or death, tumor cells metabolically reprogram and engage autophagy to survive in the hostile tumor

microenvironment (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008; Lozy & Karantza, 2012). Metabolites, oxygen concentration, and oncogenes all regulate the initiation of autophagosome formation, and the regulation of autophagy is finely balanced by the integration of all these signals (Fig. 2.3). In this section, we provide an overview of the regulation of autophagy by specific metabolites and metabolic stressors in tumor cells, focusing on cancer-relevant pathways.

3.1. Nutrient starvation

Autophagy is strongly induced in response to nutrient starvation, which is primarily controlled by mammalian target of rapamycin (mTOR). mTOR

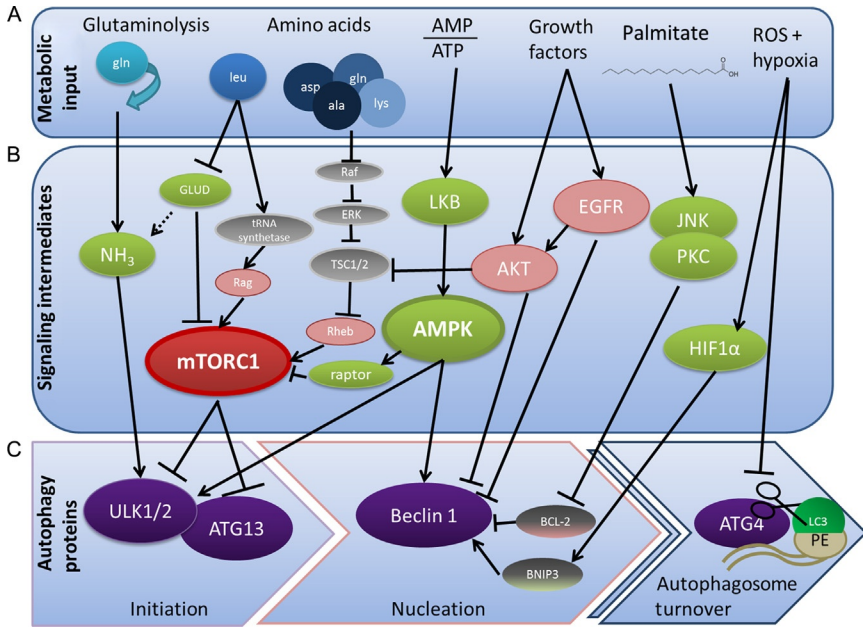

Figure 2.3 *AMPK and mTORC1 as metabolic regulators of autophagy*. The control of autophagosome formation and turnover is tightly controlled by many upstream metabolic stimuli. Metabolic input (A), such as concentrations of ammonia, general and specific amino acid levels, ATP to ADP ratio, and signals of growth and stress such as growth factors, reactive oxygen species, and palmitate, signals to initiate autophagosome formation and inhibits turnover either through signaling intermediates (B) or by directly inhibiting or activating key autophagy-related proteins (C). AMPK1 and mTOR are the main signaling integrators and modulators of autophagy—they sense glucose and amino acid levels and act on ULK1/2, ATG13, and Beclin 1 to inhibit autophagy in times of plenty and promote autophagy under energy-lean circumstances.

was initially identified as a key negative regulator of autophagy in yeast and has been confirmed to function as a major regulator of mammalian autophagy (Kroemer, Mariño, & Levine, 2010). mTOR acts as a master sensor of metabolic state; signals from growth factors, amino acids, oxidative stress, and DNA damage alter mTOR interactions with binding partners, thereby regulating mTOR activity. Active mTORC1 under nutrient conditions modulates the rates of translation, lipid synthesis, and mitochondrial proliferation and phosphorylates ULK1/2 and ATG13 to block autophagy. Under nutrient deprivation, ATG13 and ULK1/2 are dephosphorylated by an unknown phosphatase, leading to autophagosome formation (Jung et al., 2009; Jung, Ro, Cao, Otto, & Kim, 2010; Neufeld, 2010; Zoncu, Efeyan, & Sabatini, 2011).

3.2. Glucose

As noted by Otto Warburg in 1924, cancer cells preferentially utilize glycolysis over oxidative phosphorylation as a source of energy in aerobic conditions. Glycolysis is thought to provide a growth advantage by maintaining intracellular pools of metabolites for anabolism (Vander Heiden, Cantley, & Thompson, 2009). Therefore, cancer cells are more sensitive to low levels of glucose than nontransformed cells. Low glucose levels induce autophagy in a wide variety of mammalian cell types, and this regulation appears to be partially dependent on the activation of AMPK (Williams, Forsberg, Viollet, & Brenman, 2009). AMPK is activated by a high ratio of AMP to ATP (Kroemer et al., 2010). Under conditions of low intracellular energy, activated AMPK induces autophagy both by phosphorylating ULK1, resulting in its activation, and by inhibiting mTORC1 via phosphorylation of Raptor (Kim, Kundu, Viollet, & Guan, 2011; Mihaylova & Shaw, 2011). During glucose deprivation, AMPK-dependent Beclin 1 phosphorylation activates the proautophagy Beclin 1/VPS34 complex (Kim et al., 2013). However, the balance of nutrient availability is crucial for autophagy induction, especially since autophagy is an ATP-consuming process. Under starvation conditions, the addition of glucose (up to a threshold) promotes autophagy via a p38 MAPK-dependent pathway (Moruno-Manchón, Pérez-Jiménez, & Knecht, 2013).

3.3. Amino acids

Autophagy is inhibited in an mTORC1-dependent manner based on the levels of amino acids in the cytoplasm. Amino acids activate Rag GTPases,

which promote translocation of mTORC1 to the lysosomal surface, resulting in mTORC1 activation and inhibition of autophagy via ULK1/2. Intralysosomal amino acid levels also regulate mTORC1 activity in a vacuolar ATPase-dependent manner, which may function as a means of feedback inhibition of the autophagic process (Sancak et al., 2008, 2010; Zoncu, Bar-Peled, et al., 2011). Amino acid levels also alter the signaling of the RAS/RAF1/ERK1/2 pathway, which regulates autophagy induction. High amino acid levels inhibit the activation of RAF1, which prevents ERK1/2-dependent phosphorylation of Gα-interacting protein, resulting in decreased stimulus-induced autophagy in HT-29 intestinal cells (Gozuacik & Kimchi, 2004; Ogier-Denis, Pattingre, El Benna, & Codogno, 2000; Pattingre, Bauvy, & Codogno, 2003). Specific amino acids also have distinct effects on autophagy inhibition. Leucine has the strongest inhibitory effect on autophagy. Leucyl-tRNA synthetase, an intracellular leucine sensor, binds to and regulates Rag GTPase interaction with mTORC1, leading to autophagy inhibition (Han et al., 2012).

3.4. Glutamine

When glucose levels are low, cells commonly shift to glutaminolysis to maintain tricarboxylic acid (TCA) cycle ATP and NADPH production. Ammonia produced during glutaminolysis increases autophagic flux by an mTORC1-independent pathway (Cheong, Lindsten, & Thompson, 2012; Eng, Yu, Lucas, White, & Abraham, 2010). Moreover, leucine levels regulate glutamate dehydrogenase 1 (GLUD1) activity that promotes autophagy by inhibiting mTORC1 activity and modulating reactive oxygen species (ROS) levels (Lorin et al., 2013). Accordingly, the production of ammonia by GLUD1-mediated oxidative deamination of glutamate to alpha ketoglutarate may also regulate autophagy in a similar fashion to ammonia generated from glutaminolysis; however, this intriguing hypothesis requires further testing. Moreover, it is important to recognize that glutaminolysis may not always promote autophagy. Indeed, glutamine and leucine together have been reported to activate mTORC1 and therefore inhibit autophagy in a glutaminolysis-dependent manner (Durán et al., 2012), indicating that the regulation of autophagy by glutamine is sensitive to metabolic context. Glutamine depletion was reported to decrease mRNA levels of *Atg5* in wild-type MEFs, supporting the finding that glutaminolysis may promote autophagy (Lin et al., 2012).

3.5. Lipids and free fatty acids

In cancer cells, the impact of altered lipid metabolism on autophagy regulation is not as well defined as that of glucose and glutamine metabolism. Fatty acid synthesis is generally restricted to specific tissues, but is often upregulated in cancers (Santos & Schulze, 2012). Palmitate, the simplest and most abundant fatty acid and the product in fatty acid synthesis, stimulates autophagy in the muscle, liver, neurons, and pancreatic cells. Palmitate-induced autophagy is mediated by JNK1 activity and PKC activity and is independent of mTOR (Komiya et al., 2010; Martino et al., 2012; Tan et al., 2012). However, the induction of autophagy may not increase autophagic flux in pancreatic cells (Las, Serada, Wikstrom, Twig, & Shirihai, 2011), although there are conflicting data about the turnover of long-lived proteins. Further experiments, such as using GFP and mCherry-tagged LC3, will clarify this point. Additionally, the autophagic response to fatty acids may be highly tissue-specific. In hepatocytes, palmitate was found to promote apoptosis instead of autophagy, while oleate—the most abundant monounsaturated fatty acid—was found to promote autophagy via increasing ROS levels (Mei et al., 2011). The synthetic fatty acid 2-hydroxyoleic acid induced ER stress and autophagy in glioma cell lines but not a control fibroblast cell line and resulted in glioma cell differentiation (Marcilla-Etxenike et al., 2012; Terés et al., 2012). How 2-hydroxyoleic acid induces ER stress remains unknown. However, it has been shown that excess lipid storage in nonadipose tissue can cause ER stress, which increases autophagy via MTOR, JNK, and increased transcription of autophagy genes (B'chir et al., 2013; Ogata et al., 2006; Qin, Wang, Tao, & Wang, 2010; Tomohiro & Klionsky, 2007).

3.6. Hypoxia and ROS

Hypoxia and ROS, often found in the poorly vascularized tumor microenvironment, have been shown to increase autophagic flux via several mechanisms. Most directly, ROS inhibit ATG4 autophagosome turnover activity, allowing for the maintenance of lipidated LC3 necessary for autophagosome formation (Scherz-Shouval et al., 2007). Autophagy is upregulated during hypoxia via hypoxia-inducible factor 1α (HIF1α) induction of BNIP3 and BNIP3L, which binds to Beclin 1 to promote autophagy (Bellot et al., 2009). AMPK promotes autophagy independently of HIF in response to severe hypoxia. While BNIP3-regulated autophagy protects cells from death, AMPK-induced autophagy promotes cell death, pointing

to the influence of cellular context on the outcome of autophagy (Papandreou, Lim, Laderoute, & Denko, 2008).

ROS-mediated damage also likely controls autophagy. ROS damages DNA, proteins, and organelles (Wellen & Thompson, 2010), and accumulated damage and subsequent metabolic stress activate autophagic programs. In addition to starvation, JNK-mediated autophagy induction is often associated with oxidative stress (Eisenberg-Lerner & Kimchi, 2007; Kang, Zeh, Lotze, & Tang, 2011). Stress-activated JNK results in the phosphorylation of BCL-2, an antiapoptotic protein that binds to and inhibits Beclin 1 (Pattingre et al., 2005), causing the release of Beclin 1 and autophagy induction (Wei, Pattingre, Sinha, Bassik, & Levine, 2008). In another indirect mechanism, low-oxygen concentrations lead to the acidification of the environment, and autophagy is upregulated in response to low pH independent of oxygen concentration (Wojtkowiak et al., 2012).

4. AUTOPHAGY AND TUMOR SUPPRESSION

Scientific evidence supports both tumor-promoting and tumor-suppressive functions for autophagy and the exact role of autophagy during cancer progression depends on tumor type, context, and stage. Here, we discuss the genetic evidence supporting the role of autophagy-related proteins (ATGs) as tumor suppressors and review the potential mechanisms through which autophagy impairs tumor initiation and progression (Fig. 2.4).

4.1. ATGs as tumor suppressors

Genetic evidence that autophagy can prevent tumor formation first emerged through studies of *beclin 1* (Liang et al., 1999), which was found to be monoallelically deleted in 40–75% of cases of sporadic human breast, ovarian, and prostate cancer. Furthermore, mice lacking a single copy of *beclin 1* developed spontaneous lymphoma, hepatocellular carcinoma, and lung adenocarcinomas (Qu et al., 2003; Yue, Jin, Yang, Levine, & Heintz, 2003). Notably, the second allele of *beclin 1* was not lost in these tumors, further corroborating that *beclin 1* functioned as a haploinsufficient tumor suppressor. In addition, multiple Beclin 1-interacting partners have been implicated as tumor suppressors. UVRAG, a Beclin 1-interacting protein that positively regulates autophagy, is allelically deleted in human colon carcinoma (Liang et al., 2006; Liang, Feng, Ku, & Oh, 2007). Moreover, frameshift mutations in the polyadenine tract of the UVRAG gene resulting in decreased

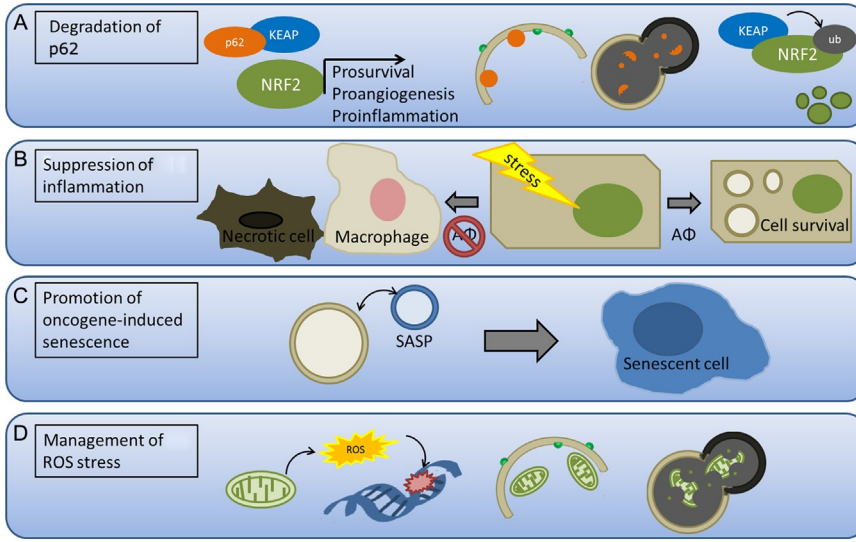

Figure 2.4 *Tumor-suppressive roles of autophagy*. (A) Autophagy prevents p62/NRF2 pathway prosurvival, proangiogenic, and proinflammatory signaling. During hypoxia, p62 binds to and sequesters KEAP, thereby preventing ubiquitination of NRF2. NRF2 can then promote transcription of prosurvival, proangiogenic, and proinflammatory genes that enhance tumor growth. When autophagy is active, p62 is degraded by sequestration within the autophagolysosome, thereby allowing ubiquitination and degradation of NRF2. (B) Autophagy prevents necrosis and inflammation by promoting survival in stressed cells. Tumor cells with decreased autophagy (AΦ) are more prone to necrosis following stress, which recruits macrophages, promotes inflammation, and fuels tumor growth. (C) Autophagy promotes oncogene-induced senescence by enhancing the senescence-associated secretory phenotype (SASP). (D) Autophagy suppresses reactive oxygen species (ROS) accumulation and genomic damage, which helps prevent genomic instability—an important driver of tumorigenesis. The mitochondria produce ROS under normal metabolic conditions and may increase the production of ROS when damaged. Mitophagy is upregulated in response to ROS and clears excess and damaged mitochondria, which then mitigates ROS production.

autophagy are present in gastric carcinomas (Kim et al., 2008). Mice lacking SH3GLB1/Bif-1, which interacts with Beclin 1 via UVRAG, exhibit a significantly higher rate of spontaneous tumors (Takahashi et al., 2007), and reduced SH3GLB1/Bif-1 expression, which correlates with decreased autophagy, is observed in gastric carcinoma (Lee et al., 2006). Besides the well-characterized oncoprotein BCL-2 interaction with Beclin 1, two other oncoproteins have been more recently shown to interact with Beclin 1 leading to autophagy suppression and oncogenesis. AKT-mediated Beclin 1 serine phosphorylation enhances its interaction with vimentin and decreases

autophagy. Depletion of vimentin or expression of a nonphosphorylatable Beclin 1 mutant in AKT-overexpressing cells increases autophagy and inhibits transformation, supporting the hypothesis that autophagy suppresses tumor initiation in AKT-driven tumors (Wang et al., 2012). EGFR-mediated Beclin 1 tyrosine phosphorylation suppresses the formation of the proautophagy Beclin 1/VPS34 complex, which may contribute to tumor progression and chemoresistance in human non-small cell lung cancer xenografts harboring oncogenic EGFR mutations (Wei et al., 2013).

In addition to Beclin 1 and its associated proteins, other ATGs have been implicated as suppressors of spontaneous tumorigenesis. Mice with systemic mosaic deletion of *Atg5* and liver-specific $Atg7^{-/-}$ mice develop liver adenomas (Inami et al., 2011; Takamura et al., 2011). *Atg4C* knockout mice exhibit increased susceptibility to fibrosarcomas in a chemical carcinogen model (Mariño et al., 2007). Mice with hematopoietic stem cell deletion of *Atg7* develop an atypical myeloproliferation resembling human myelodysplastic syndrome and acute myeloid leukemia (Mortensen et al., 2011). Frameshift mutations in *ATG2B*, *ATG5*, and *ATG9B* have been reported in gastric and colorectal carcinomas, further suggesting that the components of the core autophagic machinery act as tumor suppressors in human cancers (Kang et al., 2009).

4.2. Autophagy-dependent degradation of p62/SQSTM1

The accumulation of p62/SQSTM1, an autophagy cargo receptor, promotes tumorigenesis: Liver tumor size is reduced in $Atg7^{-/-}$ mice by simultaneous *p62* deletion (Takamura et al., 2011), *p62* gene targeting reduces anchorage-independent growth of human hepatocellular carcinoma cells (Inami et al., 2011), $p62^{-/-}$ mice fail to develop RAS-induced lung carcinomas (Duran et al., 2008), and *p62*-null cells have impaired RAS transformation (Guo et al., 2011). In KRAS-driven tumor cells, p62 activates Nrf2 and NF-κB, which stimulate proangiogenic and proinflammatory responses, respectively, thereby contributing to aggressive tumor progression. Thus, increased autophagy enhances p62 degradation, leading to diminished angiogenic and inflammatory responses (Duran et al., 2008; Kim, Hur, et al., 2011; Mathew et al., 2009).

p62/SQSTM1 activation of the Nrf2 pathway in autophagy-deficient cells is especially important in tumor progression (Komatsu et al., 2010). Notably, the Nrf2 pathway, due to inactivating somatic mutations in the E3 ubiquitin ligase Keap1, has been implicated as a survival pathway in non-small cell lung carcinomas (Singh et al., 2006). The transcription

factor Nrf2 (nuclear regulatory factor 2) regulates the expression of a wide range of genes that promote angiogenesis and facilitate cell survival. Keap1 ubiquitinates Nrf2 resulting in its degradation under normal conditions. Accumulated p62/SQSTM1 in autophagy-deficient cells directly binds to Keap1, disrupting Keap1-mediated degradation of Nrf2 and promoting aberrant Nrf2-mediated transcription (Komatsu et al., 2010). Thus, aberrant regulation of Nrf2 in autophagy-deficient cells may be an important pathway in tumor cell survival (Fig. 2.3A). Indeed, this pathway has been implicated in the spontaneous tumorigenesis of autophagy-defective liver cells (Inami et al., 2011; Takamura et al., 2011) and in the early growth acceleration of BRAF-driven lung cancers lacking Atg7 (Strohecker et al., 2013).

4.3. Autophagy prevents protumor inflammation and facilitates senescence

Because autophagy promotes tumor cell adaptation and survival during hypoxic and metabolic stress, it may suppress tumor progression by inhibiting necrosis. In solid tumors, necrotic cell death causes macrophage infiltration and proinflammatory cytokine production, and chronic inflammation generally favors cancer growth and progression (DeNardo, Johansson, & Coussens, 2008). Thus, by limiting necrosis, autophagy may actually suppress tumor growth by preventing leukocyte infiltration of the primary tumor site (Fig. 2.3B). Indeed, this ability of autophagy to restrict necrosis prevented macrophage-associated tumor inflammation and inhibited primary tumor growth in apoptosis-resistant cells (Degenhardt et al., 2006). Additionally, autophagy can facilitate the transition to senescence (Fig. 2.3C), which also prevents immune activation due to necrosis, and can lead to the elimination of premalignant cells by senescence-mediated surveillance (Kang, Yevsa, et al., 2011; Narita et al., 2011; Young et al., 2009). Autophagy allows the cancer cells to quietly survive but helps to restrict proliferation by facilitating senescence, thereby overall suppressing tumor growth.

4.4. Autophagy clears dysfunctional mitochondria

Autophagy is an important mechanism for the clearance of damaged mitochondria, a process termed mitophagy. Mitochondrial number may indirectly regulate tumor progression as the mitochondria produce ROS, which can promote tumor progression via damage to proteins or DNA

causing chromosomal instability (Ishikawa et al., 2008). In response to ROS, mitophagy is upregulated to remove excess mitochondria and mitigate ROS production (Fig. 2.3D). Increased ROS production from increased metabolic rate can damage the mitochondria, which in turn can increase metabolic stress in the cell. Accordingly, in autophagy-defective cells, metabolic stress induces more DNA damage, increased genomic instability, and increased accumulation of damaged mitochondria than in wild-type control cells (Belaid et al., 2013; Mathew et al., 2009). By clearing damaged mitochondria and controlling intracellular ROS levels, autophagy may exert a tumor suppressor function.

5. TUMOR-PROMOTING FUNCTIONS OF AUTOPHAGY

Although reduced autophagy can promote tumor development, autophagy provides cancer cells with certain selective advantages to cope with stress and promote metabolic adaptation. Hence, a basal level of autophagy appears to be necessary for the optimal survival and fitness of cancer cells. The following section provides an overview of several potential mechanisms by which autophagy may promote tumor progression (Fig. 2.5).

5.1. Autophagy and metabolic adaptation in cancer

5.1.1 Autophagy and oxidative mitochondrial metabolism

Strong oncogenic insults like RAS activation lead to increased autophagy. In pancreatic ductal adenocarcinoma (PDAC), where activating KRAS mutations are present in greater than 90% of tumors, elevated autophagy is found in both primary PDAC tumors and cell lines. Genetic inhibition of autophagy in PDAC cells potently suppresses proliferation *in vitro* and elicits robust tumor regression and prolonged survival in pancreatic cancer xenografts and genetic mouse models (Yang et al., 2011). Because RAS activation is marked by profound metabolic alterations that promote energy production and support the biosynthesis of macromolecules needed for rapid proliferation, it has been hypothesized that autophagy maintains key metabolic pathways in RAS-transformed cells. In support, the loss of autophagy during RAS transformation is associated with reduced mitochondrial oxygen consumption and decreased levels of TCA cycle intermediates (Guo et al., 2011; Yang et al., 2011). This requirement for autophagy to maintain oxidative mitochondrial metabolism of RAS-transformed cells indicates that the protumor effects of autophagy are not limited to survival functions in

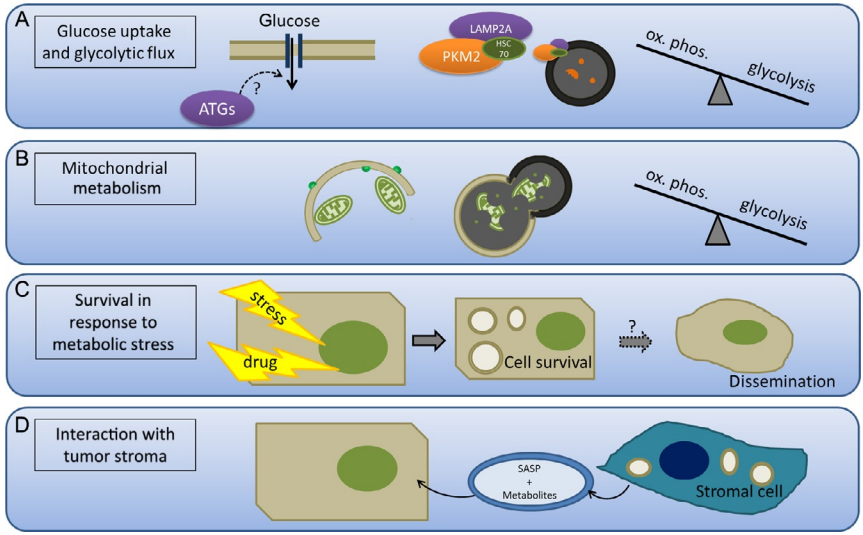

Figure 2.5 *Tumor-promoting roles of autophagy*. (A) Autophagy promotes glucose uptake and glycolytic flux. Autophagy has been shown to promote glucose uptake in cancer cells, although the mechanism remains to be elucidated. Additionally, increased chaperone-mediated autophagy (CMA) promotes degradation of PKM2, a rate-limiting glycolytic enzyme. Thus, CMA can control the rate of flux through glycolysis and whether glycolytic intermediates are used for energy production or anaplerosis. (B) Autophagy selectively degrades the mitochondria and therefore the machinery necessary for fatty acid oxidation and oxidative phosphorylation. This enhances the shift to glycolysis, which is characteristic of cancer cells. (C) Autophagy promotes survival in response to metabolic stress such as growth factor deprivation, acidic environment, and ER stress by recycling cytoplasmic material in order to maintain the basal energy state and clear damaged, misfolded proteins. This process may also be important for survival during tumor dissemination and metastasis. (D) Autophagy in stromal cells induced by the hypoxic and acidic tumor microenvironment promotes the secretion of metabolites and growth signals via senescence-associated secretion phenotype (SASP) that enhances tumor cell growth.

response to external stresses. Rather, autophagy contributes to the metabolic fitness of the entire tumor population. Remarkably, this requirement for autophagy may be oncogene-dependent, as autophagy has been demonstrated to restrict, rather than promote, proliferation driven by oncogenic PI3K in a three-dimensional mammary culture model (Chen, Eritja, Lock, & Debnath, 2013). As RAS is one of the few oncogenes that stimulate—rather than suppress—autophagy, it will be interesting to determine whether this requirement for autophagy is conserved in other oncogenic contexts.

5.1.2 Glucose metabolism

Many tumors preferentially use aerobic glycolysis, which allows for the accumulation of metabolic intermediates required for anabolism (Hsu & Sabatini, 2008). CMA and selective macroautophagy both play important roles in regulating the shift to aerobic glycolysis in cancer cells. CMA is upregulated in diverse tumor types and is necessary for tumor growth and metastasis in lung cancer cells, and inhibition of CMA decreases the rate of glycolysis characteristic of tumor growth (Kon et al., 2011). More specifically, CMA controls the levels of the metabolic enzyme PKM2 (Fig. 2.4A), which is often upregulated in many tumor types and particularly glioblastoma. The PKM2 isoform of pyruvate kinase is slower at metabolically converting phosphoenolpyruvate to pyruvate than the M1 isoform; this causes glycolytic intermediates to accumulate and drives tumor cell proliferation and growth by promoting key biosynthetic side reactions in the glycolytic pathway. CMA can selectively degrade PKM2, thereby regulating the levels of the metabolic intermediates, glucose-6-phosphate and fructose-1,6-bisphosphate, and the levels of ATP (Lv et al., 2011). Recently, PKM2-specific deletion was shown to have increased mammary tumor formation driven by *Brca-1* deletion (Israelsen et al., 2013), consistent with the notion that cancer cells prefer low pyruvate kinase activity. Therefore, the degradation of PKM2 by CMA may promote tumor progression.

The number of mitochondria present also regulates the shift to anaerobic metabolism. BRAF-driven melanoma cells decrease the rate of mitochondrial biogenesis in order to shift from oxidative phosphorylation to glycolysis (Haq et al., 2013; Ho et al., 2012; Vazquez et al., 2013). If mitophagy is aberrantly activated, decreased numbers of mitochondria shift the cells to glycolysis in a similar mechanism to BRAF regulation of mitochondrial biogenesis (Fig. 2.4B). RCAN1-1L, whose expression is increased in response to oxidative stress, can open the MPT pore and decrease ATP levels. This inhibits mTOR signaling via AMPK, resulting in increased mitophagy and a shift to glycolysis (Ermak et al., 2012). In addition to shifting the metabolic pathways to preferentially use glucose, autophagy also facilitates glucose uptake (Fig. 2.4A) and glycolytic flux in RAS-transformed cells, which is required for adhesion-independent proliferation (Lock & Debnath, 2011; Lock et al., 2011).

5.1.3 Amino acids

In addition to glucose, amino acids are necessary for cancer cell growth. In yeast, autophagic breakdown of proteins during starvation generates

cytosolic amino acid pools crucial for survival (Onodera & Ohsumi, 2005). Amino acids feed into cataplerotic pathways and can be used to maintain biosynthetic capacity in rapidly dividing cancer cells. Glutamine, the most abundant amino acid in mammalian cells, is important in cancer progression as a metabolic intermediate (DeBerardinis et al., 2008; Gaglio et al., 2011). As glycolytic rates increase, tumor cells rely increasingly on glutamine to replenish the TCA cycle and maintain ATP production (Burgess, 2013). In pancreatic cancer, glutamine feeds into glutaminolysis, utilizing steps in the TCA cycle to generate NADPH, maintain the cellular redox state, and provide metabolites for anaplerosis (Son et al., 2013). In wild-type MEFs, loss of autophagy was also found to decrease the levels of intracellular glutamine and also mimic the metabolic changes associated with glutamine depletion, indicating that autophagy normally helps to maintain intracellular stores of glutamine. However, in the same study, glutamine deprivation did not increase the levels of autophagy, and the *Atg5* mRNA level decreased (Lin et al., 2012). Therefore, how autophagy may increase specific amino acids during deprivation remains to be defined.

5.1.4 Lipids

Lipid metabolism is altered in cancer—tumor cells reactivate *de novo* lipid synthesis, ATP-citrate lyase is required for transformation *in vitro*, cholesterol synthesis in prostate cancer is increased, and fatty acid oxidation is an important source of energy for prostate cancer cells (Santos & Schulze, 2012). Autophagy in the specific form of lipophagy is important for the degradation of lipid droplets in the adipose tissue (Singh & Cuervo, 2012), and autophagy regulates lipid metabolism in hepatocytes as triglyceride hydrolysis is impaired in $Atg5^{-/-}$ cells (Singh et al., 2009). Whether these processes affect tumor lipid metabolism requires further study.

Additionally, autophagy impacts lipid metabolism by altering the mitochondrial number. *Atg7* deleted, p53 mutant cells in a KRAS-driven NSCLC model have intracellular lipid accumulation because of increased dysfunctional mitochondria that compromises fatty acid oxidation, suggesting that autophagy is crucial to maintain lipid metabolism in KRAS and p53 mutant cells. This prevents the efficient growth of tumor cells and turns them into lipid cysts instead of tumors (Guo et al., 2013).

5.2. Autophagy promotes cell survival under metabolic stress

As discussed earlier in the text, autophagy is strongly activated under periods of oxidative and metabolic stress, and depending on the extent and severity

of stress, autophagy serves to prolong cell survival in the primary tumor and possibly also during tumor dissemination and metastasis (Fig. 2.4C). In melanoma cells driven by oncogenic Ras or MEK, the removal of leucine does not induce autophagy to the same extent as nontransformed, immortalized melanocytes. The aberrant activation of mTOR via Ras prevents autophagy induction and the cells are sensitized to apoptosis, presumably because translation continues although the lost leucine is not recycled intracellularly (Sheen, Zoncu, Kim, & Sabatini, 2011). Following growth factor withdrawal, autophagy is essential for maintaining cell survival in apoptosis-deficient hematopoietic cells and can sustain viability for several weeks. IL3-deprived cells become less glycolytic and use autophagy as a catabolic process to maintain mitochondrial respiration and levels of ATP (Lum et al., 2005). Increased autophagy regulated by the PI3K/AKT/mTOR pathway prolongs cancer cell survival under acidic environment stress produced by glycolysis (Wojtkowiak et al., 2012). Autophagy also prevents ER stress-induced cell death during protein overproduction (e.g., induced by oncogenes such as Myc) by clearing excess and misfolded proteins (Tomohiro & Klionsky, 2007). Indeed, Myc-driven tumors have increased cell growth, ER stress, and metabolic rate, and autophagy inhibition enhances therapy-induced apoptosis in a Myc-driven model of lymphoma (Amaravadi, Yu, & Lum, 2007; Dang, 1999; Miller, Thomas, Islam, Muench, & Sedoris, 2012).

5.3. Autophagy in the tumor stroma

Autophagy prolongs tumor cell survival under stressful conditions. It should be noted that the acidic, hypoxic, or nutrient-starved environment also induces autophagy in the surrounding stromal cells, which promotes tumor growth (Fig. 2.4D). Serum-deprived mesenchymal stem cells induce autophagy and support MCF7 growth in xenograft models by secreting growth factors and antiapoptotic factors (Sanchez et al., 2011). While autophagy-induced senescence in cancer cells limits growth, autophagy-induced senescence in the tumor stroma may promote cancer by enhancing the senescence-associated secretory phenotype (SASP) and promoting the secretion of growth factors and cytokines that enhance tumor progression (Capparelli, Chiavarina, et al., 2012; Capparelli, Guido, et al., 2012; Capparelli, Whitaker-Menezes, et al., 2012; Maes, Rubio, Garg, & Agostinis, 2013).

In addition to modulating secretion in senescent fibroblasts, autophagy in cancer-associated fibroblasts (CAFs) may directly fuel cancer cell

metabolism. Autophagic senescent CAFs release metabolites such as glutamine, ketone bodies, and glycolytic intermediates that may promote tumor growth and metastasis. These studies raise the possibility that autophagy in the tumor stroma is important for the continued growth of the tumor (Ko et al., 2011; Martinez-Outschoorn et al., 2010; Salem et al., 2012).

5.4. Autophagy inhibition in cancer therapy

The increased dependence of tumors on altered metabolism is an attractive therapeutic target. In addition to targeting metabolic enzymes, targeting autophagy may provide a similar benefit. However, such an approach is complicated by the multifaceted role of autophagy in tumor formation and progression (Cheong, Lu, Lindsten, & Thompson, 2012). Increased autophagy has been observed in tumor cells following numerous anticancer treatments and is proposed to represent a common adaptive stress response that enables tumor cells to survive these therapeutic insults (Fig. 2.4C). This has motivated significant interest in combining autophagy inhibition with other agents to synergistically eliminate cancer cells. Readers are referred to several reviews for additional information (Amaravadi et al., 2011; Eisenberg-Lerner & Kimchi, 2009; Høyer-hansen & Jäättelä, 2008).

Notably, certain targeted therapies that enhance autophagy *in vitro* may benefit from combined autophagy inhibition. Autophagy is upregulated in response to erlotinib in NSCLC cell lines and combined treatment with chloroquine, an antimalarial that inhibits autophagy, enhances erlotinib sensitivity (Li, Lam, Mak, Zheng, & Ho, 2013). Similarly, gastrointestinal stromal tumors exhibit enhanced autophagy in response to imatinib, which lessens the therapeutic benefit. Combined inhibition of autophagy with imatinib treatment increased the number of cells undergoing apoptosis, both *in vitro* and *in vivo*, and reduced the outgrowth of resistant cells (Gupta et al., 2010). Moreover, upon treatment with the VEGF-neutralizing antibody bevacizumab, increased autophagy due to hypoxia promotes tumor cell survival and resistance to this antiangiogenic therapy (Hu et al., 2012). In contrast, inhibition of erlotinib-induced autophagy in human NSCLC xenografts *in vivo* by inducible expression of a Beclin 1 tyrosine phosphomimetic mutant resulted in partial chemoresistance (Wei et al., 2013), suggesting that the effects of autophagy inhibition may vary depending upon the autophagy step targeted, *in vitro* versus *in vivo* studies, or due to other differences in tumor type or experimental systems.

In the previously mentioned examples, autophagy is targeted due to its induction in response to therapy, but autophagy inhibition can also synergize with therapies that do not normally promote autophagic flux. For example, combining autophagy inhibition with immunotherapy could increase efficacy. Hypoxia-induced autophagy prevents lung cancer cells from cytolytic T-cell mediated cell death, but inhibition of autophagy combined with immunotherapy may provide a powerful and tumor-specific therapy (Noman et al., 2011). Another synergistic approach involves targeting the proteasome pathway and autophagy in tumor cells that are prone to ER stress. Autophagy inhibitors in combination with proteasome inhibitors increase suppression of proliferation and induce apoptosis in hepatocellular carcinoma (Hui et al., 2012). Additional studies in multiple myeloma cells also show the same increased sensitivity to the combination of proteasome inhibitors and autophagy inhibitors *in vitro* (Kawaguchi et al., 2011).

Importantly, one should recognize that many studies of autophagy inhibition as anticancer therapy have employed the lysosomal inhibitor HCQ. Hence, an important caveat for these experiments is that the cytotoxic effects of HCQ and similar agents are likely to involve processes other than autophagy. To date, the precise contributions of autophagy inhibition toward the efficacy of these antimalarials remain uncertain. Moreover, compensatory pathways, such as CMA, may influence the efficacy of autophagy inhibition as a therapeutic approach. For example, autophagy inhibition in combination with the HDACi vorinostat in a sensitive T-cell lymphoma cell line results in decreased cell death, but the resulting vorinostat-resistant subclones become partially resensitized by the inhibition of CMA (Dupéré-Richer et al., 2013).

While it remains controversial whether autophagy can mediate cell death, several studies demonstrate that genetic knockdown of autophagy blocks tumor cell death induced by oncogenic RAS (Elgendy, Sheridan, Brumatti, & Martin, 2011) or by various chemotherapeutic agents (Janku, McConkey, Hong, & Kurzrock, 2011; Notte, Leclere, & Michiels, 2011). Furthermore, acute inhibition of autophagy can limit chemotherapy responses *in vivo* by preventing autophagy-dependent anticancer immune responses (Michaud et al., 2011). Thus, additional studies are needed to further clarify the contexts in which autophagy inhibition will be beneficial in the treatment of cancer, but as these studies have shown, autophagy inhibition as a clinical therapy will not be straightforward.

6. CONCLUSION

Autophagy and metabolism in cancer cells are inexorably linked. The cross regulation of these processes acts to buffer cancer cells from the environmental and internal stresses caused by excessive proliferation. As more targeted therapies are being designed and tested, unintended consequences on autophagy, both positive and negative, must be considered in order to predict and combat side effects and resistance mechanisms. Therefore, further understanding of how autophagy contributes to cancer cell metabolism will provide insight into how to better treat cancers.

ACKNOWLEDGMENTS

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PERSPECTIVE

'Doubling down' on the autophagy pathway to suppress tumor growth

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In this issue of *Genes & Development*, Wei and colleagues (pp. 1204–1216) use elegant genetic approaches to simultaneously delete the essential autophagy gene *FIP200* (FAK family-interacting protein of 200 kDa) and the signaling adaptor p62/SQSTM1 within established murine tumors, which reveals an unexpected synergism between the autophagy pathway and p62 in driving tumor growth. Intriguingly, these observations suggest that the combined targeting of autophagy and p62 may serve as an effective approach to treat specific cancers.

Macroautophagy (hereafter called autophagy) is a catabolic process in which cells sequester cytoplasmic proteins and organelles into double-membrane vesicles, called autophagosomes, and deliver them to the lysosome, where the contents are subsequently digested and recycled. The bulk degradation of cellular material through autophagy serves as an important quality control mechanism and survival pathway during starvation and stress (Kimmelman 2011). Given the prominent role of stress resistance and survival in the development and progression of cancer, it is not surprising that the autophagy pathway is emerging as an important determinant of tumor cell fate. However, current evidence indicates multifaceted roles for autophagy during tumorigenesis, either suppressing or promoting tumor growth, depending on the cancer subtype and the stage of tumor development (Kimmelman 2011; White 2012). To characterize the distinct functions of autophagy during tumor progression, separate from its role in tumor initiation, Wei et al. (2014) developed an inducible mouse model to ablate autophagy within established tumors and uncovered that the autophagy pathway cooperates with the signaling adaptor p62/SQSTM1 to drive tumor growth.

The core components of the autophagy machinery, called ATGs (for autophagy-related), are conserved from yeast to humans. One notable exception is FIP200 (FAK family-interacting protein of 200 kDa), which lacks an obvious sequence ortholog within yeast but is critical for autophagy in higher eukaryotes. In mammals, FIP200 forms a complex with the serine–threonine kinase ULK1 and two additional proteins, ATG13 and ATG101, which together serve to regulate the early steps of autophagosome formation. Subsequently, additional ATGs mediate elongation of the autophagosome membrane and, ultimately, its closure to form the double-membrane vesicle. Selective autophagy receptors that bind LC3, of which the archetypal family member is p62/SQSTM1, are recruited with their cargo to the maturing autophagosome and sequestered from the cytosol upon closure of the double-membrane vesicle (Kimmelman 2011; Boya et al. 2013). Finally, the autophagosome fuses with the lysosome, and its contents are degraded. Importantly, disruption of essential genes within the autophagy pathway, including *FIP200*, impairs autophagosome biogenesis at its earliest stages and leads to the accumulation of substrates such as p62 (Fig. 1).

The first links between autophagy and cancer came with the discovery that *beclin1* haploinsufficiency predisposes both humans and mice to tumor development (Kimmelman 2011). Subsequent studies in genetically engineered murine models (GEMMs) of cancer have revealed that defects in the autophagy pathway promote genome damage, inflammation, and cancer cell growth (White 2012). The molecular basis for these pro-oncogenic changes in part appears to be linked to the aberrant accumulation of the selective autophagy receptor p62, a multifunctional adaptor protein at the nexus of the NF-κB, NRF2–KEAP1, and mTOR signaling pathways (Fig. 1; White 2012). However, autophagy is not purely tumor-suppressive because numerous studies indicate that pathway inhibition can impede tumor growth and sensitize cancer cells to therapy. For example, tumors with activating mutations in RAS family proteins, such as lung and pancreatic cancer, rely on the autophagy pathway for growth and proliferation (Guo et al. 2013). Similarly, in GEMMs for hereditary breast cancer that harbor deletions in *PALB2*, the partial allelic loss of *beclin1* increases apoptosis and delays tumor development in a p53-dependent manner (Guo et al. 2013). Previous studies by Guan and colleagues (Wei et al. 2011)

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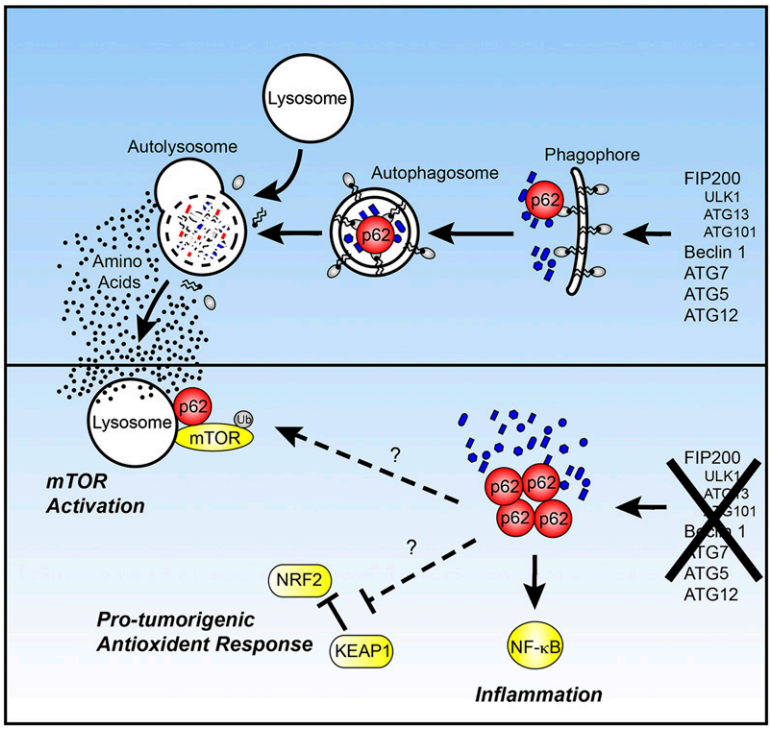

Figure 1. Autophagy regulates p62/SQSTM1 levels to control multiple pro-oncogenic signaling pathways. (*Top* panel) The formation of autophagosomes is controlled at the initiation stage by the coordinated action of the ULK1–FIP200–ATG13–ATG101 and Beclin1–Vps34 signaling complexes and enzymes, including ATG7 and ATG5–ATG12, that function to lipidate LC3. Selective autophagy receptors, including p62, interact with the autophagosome membrane and are sequestered from the cytosol along with their cargos upon membrane closure. Fusion of the autophagosome with lysosomes triggers the degradation of sequestered cargo, enabling the basic constituents of digested material to be recycled by the cells. (*Bottom* panel) Loss of essential autophagy genes, such as FIP200 or ATG7, attenuates the autophagy pathway and results in the accumulation of both selective autophagy receptors and cargo. Increased p62 potentiates NF-κB signaling and the elaboration of protumorigenic inflammatory cytokines. Defects in p62 clearance by the autophagy pathway may also reinforce pro-oncogenic signaling through the activation of mTOR and NRF2, which respectively control cellular growth and antioxidant response pathways.

have also demonstrated that genetic ablation of *FIP200* in a polyoma middle T antigen (PyMT)-driven breast cancer model profoundly inhibits the frequency and progression of murine mammary tumors . Finally, therapeutic inhibition of autophagy in diverse tumor types has shown promise as an effective anti-cancer treatment (Yang et al. 2011).

Overall, it remains unclear how autophagy inhibition leads to such seemingly disparate outcomes in different cancers. How do we determine which tumors will respond to autophagy inhibition? Fundamentally, the answers to these questions require a better understanding of how the autophagy pathway functions in specific contexts, including distinct tumor types, oncogenic backgrounds, or tumor stages. Perhaps one of the most significant challenges in assessing the function of autophagy in cancer has been an inability to discern between the contributions of autophagy to tumor initiation versus its role in tumor progression. To tackle this important issue, Wei et al. (2014) developed a clever genetic approach that allowed them to specifically ablate autophagy in advanced tumors. Mouse embryo fibroblasts (MEFs) derived from *FIP200* floxed mice were transformed with E1A/H-Ras^{V12} and subsequently transduced with a retroviral vector encoding a tamoxifen-inducible form of the Cre recombinase (Cre^{ERT}); as a result, *FIP200* could be conditionally deleted in established tumors in vivo upon administration of tamoxifen. Intriguingly, upon *FIP200* deletion and impairment of the autophagy pathway in established tumors, Wei et al. (2014) observed a profound reduction in tumor growth. These observations were not exclusive to transformed MEFs, since deletion of *FIP200* within tumors derived from PyMT transformed mouse mammary tumor cells also resulted in impaired growth. An assessment of the proportion of replicating and apoptotic cells within the two treatment groups revealed that the defect in tumor growth was a manifestation of both attenuated tumor cell proliferation and increased cell death. Although the investigators did not examine the status of metabolic pathways in the *FIP200*-null tumors, it is tempting to speculate that the impaired proliferation and increased apoptosis are at least partly associated with a failure to sustain energy and biosynthetic pathways (Guo et al. 2013).

Similar to previous studies examining the function of autophagy during tumorigenesis, Wei et al. (2014) observed a marked accumulation of p62 upon deletion of *FIP200* (Mathew et al. 2009; White 2012). However, in stark contrast with those reports, p62 accretion in FIP200-deficient tumor cells correlated with reduced tumor growth, not enhanced tumorigenesis. To determine the mechanisms underlying this discrepancy, the investigators RNAi-depleted this signaling adaptor in *FIP200*-null cells to assess how cotargeting these molecules impacted tumor growth. Remarkably, p62 knockdown in *FIP200*-null tumors further impaired tumor growth compared with *FIP200* alone. Thus, in certain circumstances, autophagy synergizes with p62 to promote tumor cell survival or expansion. To validate this result, Wei et al. (2014) performed a rescue of p62 expression in p62-deficient, *FIP200*-null tumor cells, which led to a partial restoration of tumor growth. Collectively, these observations highlight a previously unknown collaboration between the FIP200 pathway and p62 that can promote the growth of established tumors.

Although the observations of Wei et al. (2014) suggest that autophagy synergizes with p62 to drive tumorigenesis,

it is conceivable that they merely highlight a unique relationship between FIP200 and tumor growth. Hence, the investigators analyzed the effects of autophagy upregulation on tumor growth by conditionally overexpressing a constitutively active form of the transcription factor EB (TFEB^{S142A}), a master transcriptional regulator of molecules that coordinately promote both autophagy and lysosome biogenesis (Settembre et al. 2013). Congruent with a protumorigenic role for autophagy in this system, TFEB^{S142A} overexpression significantly enhanced the growth and survival of tumor cells in vivo. However, given the known effects of TFEB^{S142A} on lysosome biogenesis, it remains unclear whether enhanced tumor progression is the specific result of enhanced autophagy.

The precise mechanism by which p62 promotes growth in the absence of autophagy is a point of significant importance, particularly because dual targeting of autophagy and p62 may be an effective treatment for some cancers. To provide mechanistic insight into the tumor-promoting function of p62, Wei et al. (2014) examined the impact of NF-κB suppression on *FIP200*-null tumor growth. Indeed, p62 has been shown to be necessary for activation of the NF-κB pathway, and these functions have been reported to be important contributors to tumorigenesis (Duran et al. 2008; Mathew et al. 2009). However, expression of the IκBα super repressor, a dominant-negative inhibitor of the NF-κB pathway, within *FIP200*-null tumors only modestly reduced tumor growth. Thus, other functions of p62 are also likely to be important for its protumorigenic role in the context of *FIP200* ablation. One intriguing possibility is that accumulation of p62 promotes tumor growth by potentiating mTOR signaling (Fig. 1). Recently, p62 was found to facilitate the activation of mTORC1 by promoting its ubiquitination and lysosomal translocation (Duran et al. 2011; Linares et al. 2013). Given the role of mTOR in coordinating cell growth and its frequent dysregulation in cancer, it is attractive to speculate that the tumor-promoting functions of p62 may be partly rooted in this pathway. In addition, p62 regulation of NRF2–KEAP1 and the antioxidant defense pathway may also facilitate tumorigenesis in the absence of functional FIP200 (Fig. 1; DeNicola et al. 2011). Clearly, the tumor-promoting mechanisms of p62 in autophagy-deficient cells remains a critical area of future investigation.

Finally, it is important to consider the therapeutic potential of simultaneously targeting autophagy and p62 within established cancers. Although Wei et al. (2014) justified such a combinatorial approach in certain scenarios, ultimately, the availability of pharmacological inhibitors to efficiently target these pathways and further understanding of the contexts that maximize treatment efficacy will govern its therapeutic utility. In the first regard, pharmacological autophagy inhibitors such as hydroxychloroquine are already in clinical trials (Yang et al. 2011). In contrast, p62 inhibitors still need to be developed. Nevertheless, the advent of personalized medicine and an explosion in capabilities in GEMMs should make the task of identifying cancer subtypes and stages that are suitably targeted by autophagy and p62 inhibitors far less challenging than it once appeared. Gradually, we are "stacking the deck" with therapeutic options to most effectively target autophagy for cancer treatment.

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